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17241. Observations on the Formation of Connective Tissue Fibers.*

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Some information on the origin and development of collagenous fibers has been obtained from the study of cultured tissues[1-4] and wound healing.[5] In such material fine fibrils can be observed to develop between the cells. These subsequently increase in number and finally coalesce to form larger fibers. It is generally thought that the fibrils are organized from an intercellular protein that is produced by or at least influenced by the surrounding cells, but the precise mechanism is not understood.

One approach to the examination of this problem is by way of direct observations on the early submicroscopic sequences involved in fiber formation. To that end we have utilized the combined methods of electron microscopy and tissue culture.

The material studied has included fibrous arrays formed *in vitro* in association with explants of chick embryo skin and foregut, rabbit thymus and rat pericardium.‡ In preparation for microscopy the cultures containing these arrays were washed in a balanced salt solution, fixed briefly over vapors of OsO_4 and thereafter mounted on electron microscope screens by the same technics that are used for cells.[6]

Only areas of the culture in which the plasma clot had been completely lysed were suitable for microscopy. In such areas the presence or absence of connective tissue fibers could be determined with the light microscope. Generally the cell population was sparse. Some units appeared as macrophages and fibrocytes and were spread out quite thinly on the coverglass whereas others of unknown nature remained rounded up and were dispersed over the surface of the fiber mat.

As was more of less expected, it was found that these fibrous mats contain a great many more fibers than are apparent with the light microscope. In fact, the predominant type, hereinafter referred to as unit fibers, has a diameter usually less than 500 Å (Fig. 1 to 5). They are long slender strands which, over the

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† Studies made during the tenure of a fellowship in Cancer Research of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

1 Maximow, A., *Centr. allg. Path.*, 1929, **43**, 145.

2 McKinney, R. L., *Arch. exp. Zellforsch.*, 1929, **9**, 14.

3 Momigliano-Levi, G., *Z. Zellforsch.*, 1932, **16**, 389.

4 Bloom, W., and Santstrom, R. H., *Anat. Rec.*, 1935, **64**, 1.

5 Stearns, M. L., *Am. J. Anat.*, 1940, **67**, 55.

‡ All micrographs used for illustrations are of fibers that had developed from explants of chick embryo skin after 9 days in culture.

6 Porter, K. R., Claude, A., and Fullam, E., *J. Exp. Med.*, 1945, **81**, 233.

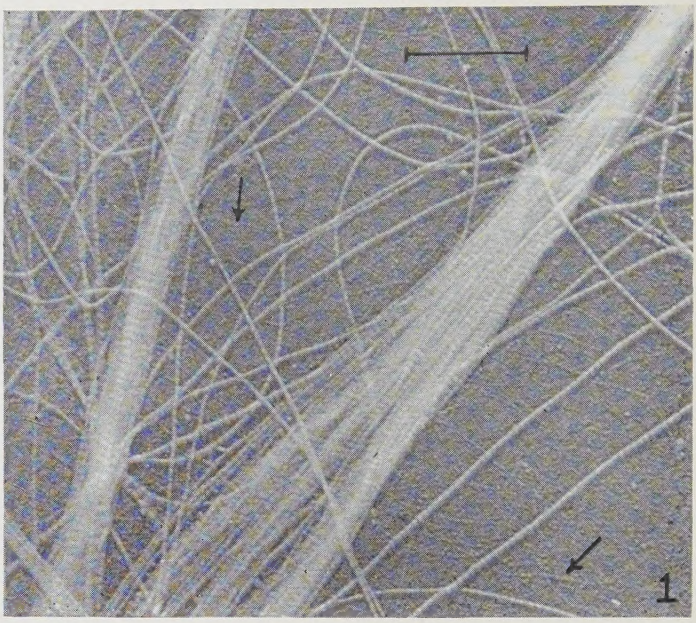

FIG. 1.
Micrograph showing numerous unit fibers and two large collagen-like strands made up of unit fibers. Beaded protofibrils are present in the background and indicated by arrows. Specimen lightly shadowed with gold, 12° angle. Mag. 15,734.

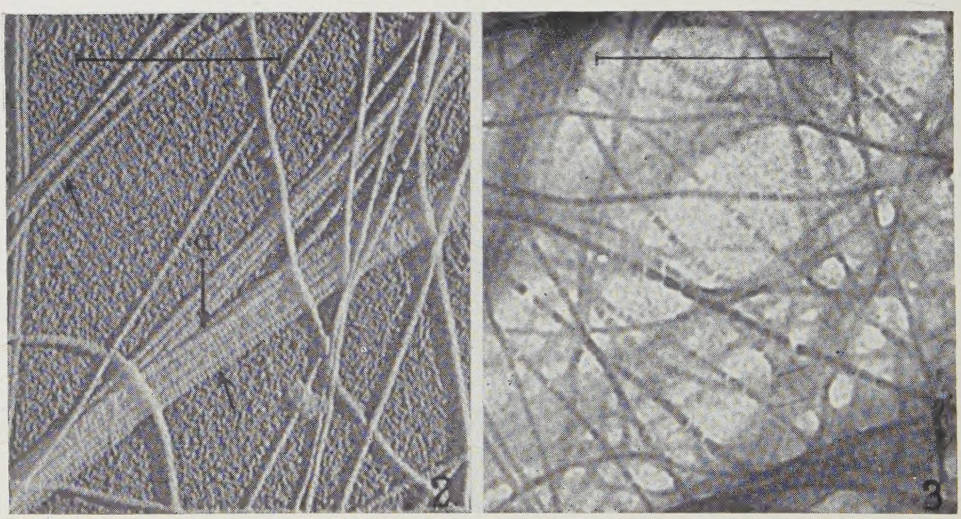

FIG. 2. Micrographs of gold-shadowed preparation showing banded structure of unit fibers (arrows). The periodicity is approximately 270 Å. At (a) there is some evidence of the development of the larger 640 Å period of collagen. Mag. 26,900.

FIG. 3. Micrograph of unit fibers in which the bands or striae are grouped in three's to form the repeating axial pattern of collagen. The intra-period bands are observed to be unequal in size and density. Mag. 30,500.

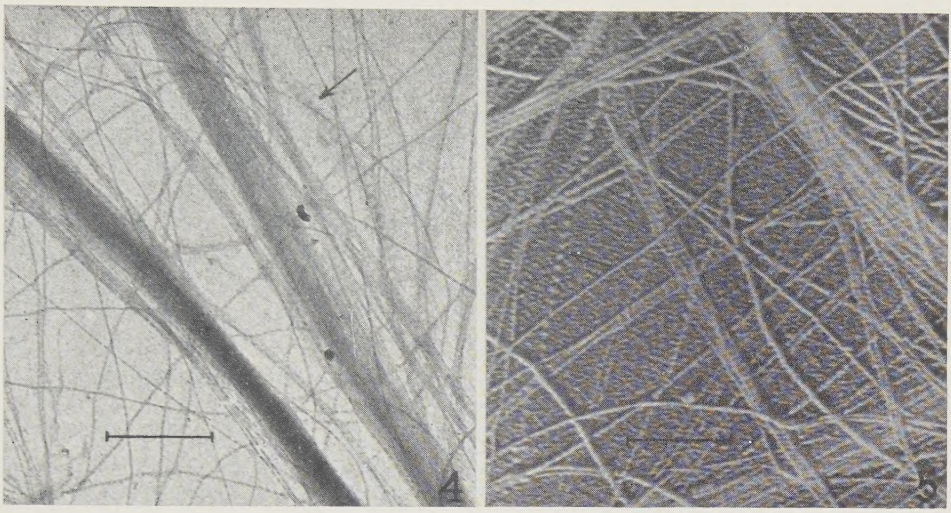

FIG. 4. Micrograph showing large-period component (arrow) of preparations organized as a fiber with densities spaced uniformly at approximately 100 Å. Mag. 13,700.
FIG. 5. Micrograph of similar material (arrow) shadowed with gold, 12° angle. Mag. 14,000.

entire width of a single field of a micrograph (12 μ), may not vary appreciably in width. At their ends they taper off gradually into slender threads 50 Å or less in diameter. The unit fibers are all striated or banded, but there is some variation in the organization of the striae or bands. In many fibers, and more especially in those of small diameter, the striae are of uniform size and evenly spaced at approximately 270 Å (Fig. 2). In other unit fibers, which are the larger and presumably more completely formed, the striae are compressed into groups of 3 which have an over-all length of 650 to 800 Å (Fig. 3). When thus grouped, the bands show some differences, 2 appearing larger and denser than the third. The pattern of these bands is repeated in each group or major period along the fiber axis so that not only each period but the whole fiber is polarized (Fig. 2 and 3).

The larger strands of the preparations—those that can be seen with the light microscope—are compound in structure (Fig. 1 to 5). They are composed of varying numbers of unit fibers, and hence, vary in diameter. The size most frequently encountered in these preparations is 0.5 μ or less. Usually these strands, when not stretched, show the characteristic periodicity of collagen[7,8] (640 Å) with striations extending across a part or all of the bundle. The typical collagen-like striation of these compound strands shows 2 prominent intra-period striae and one less prominent, in each repeating pattern. The more dense striae correspond to the double elevation apparent in the image of the metal-shadowed, dried fiber.

In part of the preparations where areas of the supporting plastic membrane are exposed, a fine fibrous component is frequently encountered. This consists of very slender filaments or protofibrils, 50 to 100 Å in diameter, which are similar to the ultimate tapered ends of the unit fibers. They are usually beaded. The distance between the bead varies considerably, but not infrequently it is about 270 Å, giving them a periodicity similar to that in small unit fibers. Presumably these protofibrils represent the primary association of the collagen macromolecules.

Neither these fine filaments nor the larger unit fibers have been observed in the material examined to arise as formed structures from cells.

There is a fourth component of these preparations that appears as aggregations of even-

7 Schmitt, F. O., Hall, C. E., and Jakus, M., *J. Cell. and Comp. Physiol.*, 1942, **20**, 11.

8 Gross, J., and Schmitt, F. O., *J. Exp. Med.*, 1948, **88**, 555.

ly-spaced parallel bands or densities. The organization of these is usually in the form of a fiber (Fig. 4 and 5), but occasional patches of irregular outline have been observed. The periodicity displayed measures as a rule between 1000 to 1100 Å. The densities in shadowed preparations appear as prominent elevations and there may be extremely little if any material connecting them. There is no evidence of intra-period banding. In most of the collagen preparations examined, this component has been found scattered rather sparsely among the fibers, but it has also been observed without associated fibers. We are therefore unable to decide whether it is related in any way to collagen formation, but are inclined to the view that it represents a component of the connective tissue ground substance.

The available evidence indicates that the major fibrous components of these preparations, exclusive of the last, are collagen. The periodicity of the large compound fibers is like that of collagen, the fibers resist tryptic digestion, and staining technics have identified collagen fibers in cultures paralleling these used for electron microscopy.

The sequence of events leading to mature fiber formation, while not directly observable, can be reasonably reconstructed from the character and associations of the various components of any fibrous array. Thus the relation of the unit fibers to the larger strands is clear; they are obviously the component units and appear to have come together into these parallel arrays much the same as unit fibers of fibrin aggregate to produce the larger strands of the formed clot.[9] But the origin of the unit fibers is less evident. They are apparently not spun off the cells. Instead, it is probable that they are formed, as in fibrin, through a lateral association of several protofibrils and a progressive deposition of molecular collagen on their surfaces.[10] Since the more slender unit fibers show a fine even periodicity, it appears that the larger collagen pattern of the mature fiber is a secondary development. These and other phenomena of connective tissue fiber formation will be considered more completely in a later report.

[9] Hawn, C. V. Z., and Porter, K. R., *J. Exp. Med.*, 1947, **86**, 285.

[10] Porter, K. R., and Hawn, C. V. Z., *J. Exp. Med.*, 1949, **90**, 225.

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17242. The Cellular Transfer of Cutaneous Hypersensitivity to Tuberculin in Man.

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It has been demonstrated by Chase[1] that transfer of specific cutaneous hypersensitivity of the "delayed type" to tuberculin can be accomplished in unsensitized guinea pigs by the injection of leucocytes isolated from peritoneal exudates produced in sensitized guinea pigs.

It is the purpose of this report to describe the transfer of cutaneous hypersensitivity to tuberculin in man by the intradermal injection of viable leucocytes isolated from the peripheral blood of non-tuberculous humans.

Materials and Methods. Utilizing the method of Minor and Burnett[2] it has been possible to isolate and concentrate viable leucocytes from human blood by the addition of bovine fibrinogen, Fraction I (Armour), which accelerates the rate of erythrocyte sedimentation leaving a plasma suspension of leucocytes in the supernatant portion.

[1] Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 134.

[2] Minor, A. H., and Burnett, L., *J. Hematology*, 1948, **7**, 799.

1. *Technic of obtaining leucocytes.* Whole venous blood was drawn and placed in sterile potato tubes containing 0.4 ml of a 100 mg % heparin solution for each 10 ml of blood. The tubes were inverted twice and 1.0 ml of sterile Seitz filtered bovine fibrinogen, Fraction I (Armour) solution, (containing approximately 45 mg fibrinogen per ml) was added for each 10 ml of blood and the tubes inverted twice. The heparinized and fibrinogenized blood was then transferred in 10 ml aliquots to conical centrifuge tubes which were placed in a water bath at 37°C for 1 hour. At this time the erythrocytes have become packed in the lower half of the tube and a suspension of leucocytes is contained in the supernatant plasma. The latter was transferred by capillary pipette to specially constructed 10 ml capillary-tip centrifuge tubes (Machlett, No. A 17-210) and centrifuged in an angle centrifuge at 3000 RPM for 1 hour. The cell-free plasma was then decanted and the packed leucocytes (average volume packed wet cells from each 10 ml plasma suspension = 0.025 ml) were resuspended and washed in either 2.0 ml of Tyrodes solution (pH 7.8-8.3) or 2.0 ml of freshly drawn serum obtained from the tuberculin negative recipient of the cells. The cell suspensions were collected from each tube and pooled in one capillary-tip centrifuge tube to a volume of 10 ml and centrifuged again at 3000 RPM for 30 minutes. The supernatant portion was decanted and the washing and centrifugation of the leucocytes was repeated. The supernatant portion of the second washing was decanted, the volume of packed leucocytes read, and then resuspended in either 1.0 ml of Tyrodes solution or 2.0 ml of serum. This suspension of twice washed leucocytes was then injected intradermally into the tuberculin negative recipient.

The interval between the withdrawal of blood and the injection of leucocytes into the recipient was usually not greater than 6 hours. The leucocytes were not refrigerated or stored and rigid sterile technics were observed in the procedure described.

2. *Dosage of leucocytes and vehicle for suspension.* Either 60 ml or 100 ml of venous blood was obtanied from each tuberculin positive donor with normal total peripheral and differential leucocyte count.

The average volume of packed wet leucocytes isolated from 60 ml whole blood was 0.07 ml. The leucocytes were twice washed with 10 ml of Tyrodes solution (pH 7.8-8.3) and resuspended in 1.0 ml of Tyrodes solution for intradermal injection.

The average volume of packed wet leucocytes isolated from 100 ml whole blood was 0.20 ml. The leucocytes were twice washed with 10 ml of serum and resuspended for intradermal injection in 2.0 ml of serum freshly drawn from the tuberculin negative recipient.

3. *Tuberculin Materials Used in Test.* The subjects were tested with Old Tuberculin (O.T.) or Purified Protein Derivative (PPD). The leucocyte donors reacted to either 0.1 ml (1.0 mg) O.T. or to 0.1 ml (0.005 mg) PPD intradermally with ++++ to ++++ reactions after 48 hours. The recipients of the leucocytes had no reaction to either O.T. (1.0 mg) or PPD (0.005 mg) intradermally after 48 hours.

4. *Selection of Donors and Recipients.* The group is comprised of 11 tuberculin negative recipients and 5 tuberculin positive donors. The donors and the recipients of the leucocytes were patients hospitalized on the wards of the Third (NYU) Medical Division of Bellevue Hospital or were normal young adults and ranged in age from 21 to 65 years. They were not suffering from any intercurrent infection, were neither cachectic nor myxedematous and had no clinical, laboratory or roentgenographic evidence of active pulmonary or systemic tuberculosis.

5. *Method of Leucocyte Transfer.* Two methods of leucocyte transfer were used.

a) *Prausnitz-Küstner Passive Transfer of Leucocytes.* Using the passive-transfer method of Prausnitz-Küstner, 1.0 ml of a Tyrode suspension of viable leucocytes obtained from a tuberculin positive donor was injected intradermally into the flexor surface of the forearm of the tuberculin negative recipient. After an interval of 18, 24 or 48 hours the reaction at the site of cell transfer was

TABLE I.
Cellular Transfer of Cutaneous Tuberculin Hypersensitivity to Tuberculin Negative Recipients, Using the Method of Prausnitz-Küstner.

Tuberculin negative recipient	Tuberculin reaction*† at WBC site—48 hr	Tuberculin reaction distant from WBC site—48 hrs	Duration of induced tuberculin positive state	Tuberculin status of recipient at present
S.D.	++++	+++	>2 mo.	Unknown
E.K.	++++	+++	1 "	Negative
A.G.‡	+++	+++	1 "	"
A.G.‡	Not challenged at WBC site	++	1 wk	"
S.P.	++	+++	2 "	"
N.P.	++	++++	>1 mo.	Unknown

* *Criteria for reading the intradermal tuberculin reaction:* The criteria for reading the intradermal tuberculin reaction were the same for all individuals studied:

(a) Tuberculin Negative Reaction: Recorded when no reaction occurred at the intradermal site of P.P.D. 0.1 ml (0.005 mg) or O.T. 0.1 ml (1.0 mg) after 48 hours.

(b) Tuberculin Positive Reaction: Graded according to the severity of the reaction at the intradermal site of P.P.D. 0.1 ml (0.005 mg) or O.T. 0.1 ml (1.0 mg) after 48 hours, as outlined below:

\+ —reactions more than 5 mm and not exceeding 10 mm in diameter, showing some redness and definite edema.

++ —reactions more than 10 mm but not exceeding 20 mm in diameter, with an area of redness and edema.

+++ —reactions more than 20 mm but not exceeding 30 mm with marked redness and edema.

++++—reactions exceeding 30 mm in diameter with marked redness and edema.

† The tuberculin preparation used throughout this series of observations was Old Tuberculin (O.T.).

‡ Same tuberculin negative recipient.

measured and the cell site and a control site some distance from the latter were challenged with 0.1 ml (1.0 mg) O.T. intradermally. Reactions were read in both sites at 15 and 30 minutes and 24, 48, 72 and 96 hours. Three subjects were given additional control intradermal injections of an equal volume of erythrocytes and of serum from the same donor and the sites challenged as above.

b) *Method of Tuberculin Challenge at a site distant from the cell site.* A 2.0 ml serum suspension of leucocytes was injected intradermally in the deltoid area of the tuberculin negative recipient and after an interval of 18 hours, only the skin of the forearm was challenged with 0.1 ml (0.005 mg) PPD intradermally. Readings of the tuberculin reaction in the forearm were made as outlined above.

6. *Control Observations.* Three subjects were given leucocytes from tuberculin negative donors and challenged with tuberculin in the manner described immediately above. These subjects remained tuberculin negative, subsequently were then given leucocytes from tuberculin positive donors.

The first recipient, S.D., became tuberculin positive at the site of leucocyte transfer but not in the control site prepared with donors serum nor in a site distant from both above sites. After a 16 day interval S.D. was given a second injection of leucocytes from the same tuberculin positive donor and 24 hours later the site of earlier leucocyte transfer, which had faded entirely, the new site of leucocyte transfer and a site distant from both, were each challenged with tuberculin. As is shown in Fig. 1, this recipient developed positive reactions to tuberculin in the old cell site, the new cell site and for the first time in a site distant from the cell sites. This was the first indication that a systemic as well as a local alteration of cutaneous reactivity to tuberculin occurred following leucocytic transfer.

All of the leucocyte recipients who received control intradermal injections of serum and of erythrocytes from tuberculin positive donors, developed positive reactions at the site of leucocyte transfer but not at the sites of serum or of erythrocyte transfer when each site was challenged with O.T. (1.0 mg). The negativity at the sites of serum and erythrocyte transfer persisted until general cutaneous hypersensitivity to tuberculin appeared.

Three recipients (B.H., M.P., R.G.) were injected intradermally with leucocytes obtained from tuberculin negative donors in

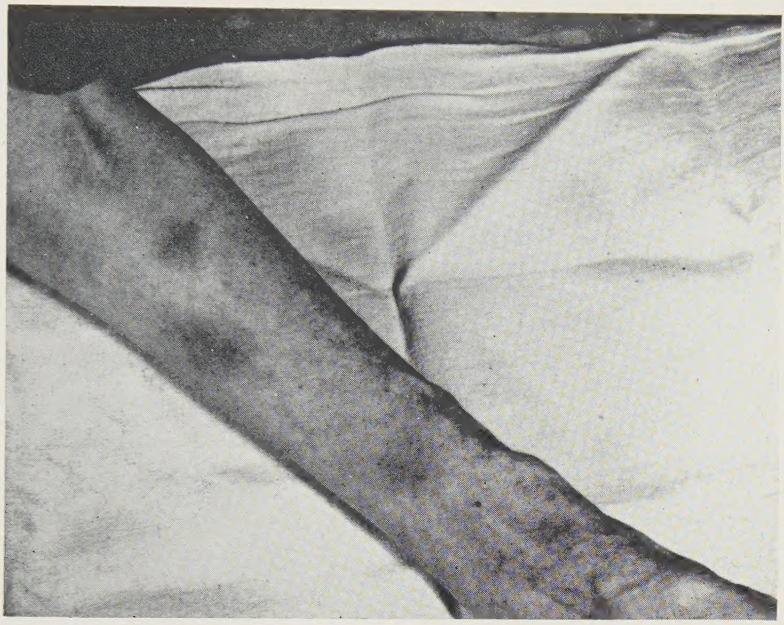

FIG. 1.
Induced Tuberculin Positive Reaction.
Subject S.D. at 24 hr.
Upper: Old cell site challenged with O.T. 1.0 mg.
Middle: New cell site challenged with O.T. 1.0 mg.
Lower: Distant site challenged with O.T. 1.0 mg.

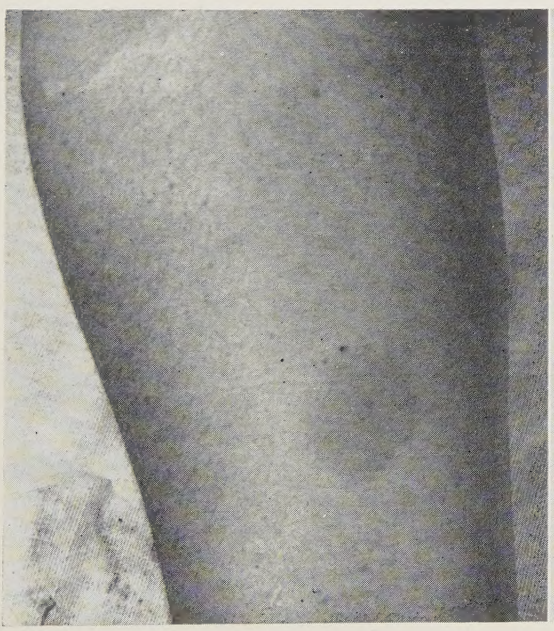

FIG. 2a.
Induced Tuberculin Positive Reaction.
Subject E.K. at 24 hr.
Site distant from cell site challenged with O.T. 1.0 mg.

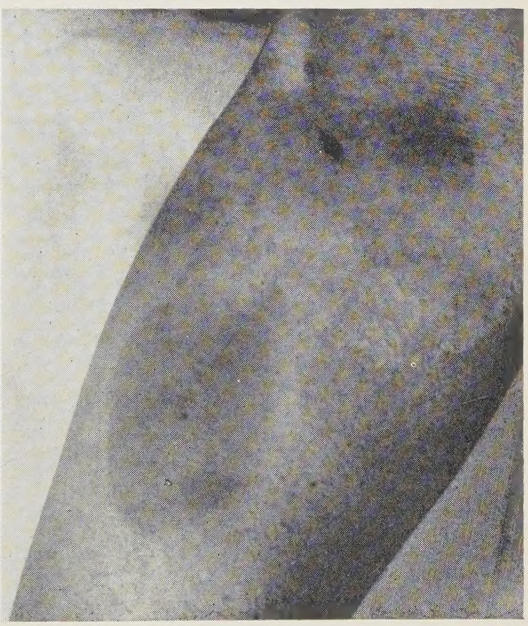

FIG. 2b.
Induced Tuberculin Positive Reaction.
Subject E.K. at 24 hr.
Cell site challenged with O.T. 1.0 mg.

TABLE II.

Cellular Transfer of Cutaneous Tuberculin Hypersensitivity to Tuberculin Negative Recipients, Using the Method of Tuberculin Challenge at a Site Distant from the Cell Site.

Tuberculin negative recipient	Tuberculin* status of leucocyte donor	Volume of packed WBC, ml	Tuberculin reaction after WBC transfer at 48 hours	Duration of induced tuberculin positive state	Tuberculin status of recipient at present
B.H.	Negative	0.04	0		
	Positive	0.05	0		
	"	0.10	+	4 days	Negative
M.P.	Negative	0.04	0		
	Positive	0.05	0		
	"	0.10	++	3 mo.	"
R.G.	Negative	0.20	0		
	Positive	0.20	++++	Unknown	Positive
P.O.	"	0.15	+++	>1 mo.	"
S.S.	"	0.20	+++	>1 "	"
A.S.	"	0.20	++	>1 "	"

* The tuberculin preparation used throughout this series of observations was Purified Protein Derivative (PPD).

order to determine whether the presence of the cutaneous tuberculin hypersensitivity of the leucocyte donor is necessary for the successful transfer of that hypersensitivity to the recipient. In each instance the passive transfer of leucocytes obtained from a tuberculin negative donor had no effect upon the tuberculin reaction of the tuberculin negative recipient. However, the subsequent passive transfer of leucocytes obtained from tuberculin positive donors given to the same three tuberculin negative recipients was followed by the development of cutaneous tuberculin hypersensitivity in each.

It appeared possible but unlikely, that the manipulation or the materials used in the isolation and concentration of the leucocytes, may have directly caused or indirectly contributed to the transfer of cutaneous hypersensitivity to tuberculin. To explore this possibility, leucocytes were obtained from a tuberculin negative donor (M.P.) and the leucocyte suspension injected intradermally into the same tuberculin negative donor (M.P.). This procedure had no effect upon the cutaneous reaction to tuberculin in this individual, whereas the subsequent transfer of leucocytes obtained from a tuberculin positive donor was followed by the development of tuberculin hypersensitivity.

The observations made on subjects (B.H.) and (M.P.) suggest the probable importance of the actual amount or dosage of the packed leucocytes transferred, in determining the development of tuberculin hypersensitivity in the individual recipient. The volume of packed leucocytes obtained from a +++ tuberculin positive donor was 0.1 ml, half of which (0.05 ml) was given to tuberculin negative recipient (B.H.) and half (0.05 ml) given to tuberculin negative recipient (M.P.). Both recipients remained tuberculin negative when subsequently challenged with tuberculin. The transfer of leucocytes obtained from another +++ tuberculin positive donor was repeated, this time 0.1 ml of packed cells was injected into each recipient (B.H. and M.P.). Subsequent challenge with tuberculin resulted in the development of a positive reaction in each recipient.

Of interest is the phenomenon observed in subject P.O. (Fig. 3). This tuberculin negative recipient had no reaction to PPD (0.005 mg) for 72 hours and then 6 hours after the transfer of leucocytes from a tuberculin positive donor, developed a positive reaction at the formerly negative PPD site, which reached its maximum intensity (++) at 48 hours. When challenged with PPD (0.005 mg) 24 hours after leucocyte transfer, this individ-

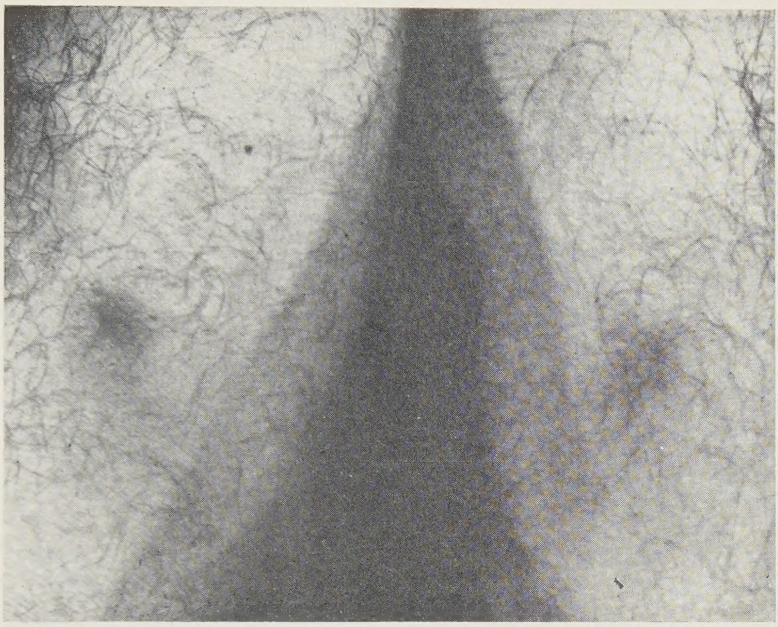

FIG. 3.
Induced Tuberculin Positive Reaction Subject P.O.
Left: Reaction at 48 hours (PPD-0.005 mg) at site distant from cell site, which had been challenged 24 hours after leucocyte transfer.
Right: Reaction at 72 hours (PPD-0.005 mg) at site distant from cell site, which had been negative until 6 hours after leucocyte transfer.

ual developed a positive reaction at the new PPD site which reached its maximum intensity (+++) at 48 hours. The delayed appearance of a positive reaction at the site of a formerly negative tuberculin test has been commented upon by several observers[3] and it usually occurs secondary to the development of a primary tuberculous infection in the interim between testing and the appearance of the positive reaction. There has been no clinical or roentgenographic evidence that a primary tuberculous infection had occurred in subject (P.O.). To substantiate this, is the observation that the degree of his induced cutaneous hypersensitivity to tuberculin has decreased progressively in intensity from a (+++) to a (+) reaction in the month following leucocyte transfer.

The observations tabulated in Table III suggest the probable importance of the degree of tuberculin hypersensitivity of the leucocyte donor, in determining the degree of induced tuberculin hypersensitivity of the individual recipient.

Of the 6 tuberculin negative recipients who were followed with repeated tuberculin testing after conversion to the tuberculin positive state, 5 spontaneously reverted to tuberculin negativity after a variable period of 4 days to 3 months.

One of the subjects (A.G.) with induced cutaneous hypersensitivity to tuberculin who spontaneously reverted to a tuberculin negative state, was sensitized again with leucocytes obtained from another tuberculin positive donor and subsequently became tuberculin positive for the second time. This subject has since become tuberculin negative again.

The recipients P.O., S.S., A.S. and R.G., with induced cutaneous tuberculin hypersensitivity of more recent origin, are still tuberculin positive at present. However, the degree of tuberculin hypersensitivity of P.O. and S.S. has decreased progressively in intensity from a (+++) to a (+) reaction in the month following leucocyte transfer. The observations

[3] Daniels, M., *Lancet*, 1943, **245**, 600.

TABLE III.
Degree of Induced Tuberculin Hypersensitivity in Relation to Degree of Hypersensitivity of Leucocyte Donor.

Tuberculin positive donor	Degree of tuberculin hypersensitivity of donor	Tuberculin negative recipient per donor	Degree of tuberculin hypersensitivity in recipient following WBC transfer	Duration of tuberculin positive state	Tuberculin status at present
		S.D.	+++	>2 mo.	Unknown
		E.K.	+++	1 ''	Negative
D.M.	+++	B.H.	+	4 days	''
		M.P.	++	3 mo.	''
		A.G.†	+++	1 ''	''
C.S.	++++	S.P.	+++	2 wk	''
		N.P.	++++	>1 mo.	Unknown
P.B.	++++	A.G.†	++	1 wk	Negative
		P.O.	+++	1 mo.	Positive*
R.H.	++++	S.S.	+++	1 ''	'' *
		A.S.	++	1 ''	'' *
		R.G.	++++	Unknown	'' *

* Each recipient still under observation.
† Same tuberculin negative recipient.

made on the recipients with induced cutaneous tuberculin hypersensitivity, who spontaneously reverted to a tuberculin negative state, indicated that the progressive decrease in the intensity of the tuberculin reaction was followed shortly thereafter by the return to tuberculin negativity.

Discussion. In twelve consecutive instances the tuberculin negative recipients of viable leucocytes obtained from tuberculin positive donors, subsequently developed cutaneous hypersensitivity to tuberculin. When the leucocytes were obtained from tuberculin negative donors, cutaneous hypersensitivity to tuberculin did not subsequently develop in the tuberculin negative recipient.

The cutaneous hypersensitivity to tuberculin following leucocyte transfer is a transient phenomenon of 4 days to 3 months duration. The induced cutaneous hypersensitivity to tuberculin can be produced again in the same individual.

The degree of induced hypersensitivity to tuberculin seems to bear a relationship to the dosage of leucocytes used and the degree of tuberculin hypersensitivity of the leucocyte donor.

These observations in man are in agreement with those made by Chase[1] in the guinea pig.

Summary and conclusions. 1. It has been possible to passively transfer in 12 consecutive instances cutaneous tuberculin hypersensitivity to tuberculin negative human recipients by means of an intradermal injection of viable leucocytes obtained from the blood of tuberculin positive human donors.

2. The effort at the passive transfer of cutaneous tuberculin hypersensitivity was unsuccessful when the leucocytes used in the transfer were obtained from tuberculin negative donors.

3. The induced cutaneous hypersensitivity to tuberculin is a transient phenomenon of 4 days to 3 months duration, which can be produced again in the same individual.

4. The degree of induced hypersensitivity to tuberculin seems to bear a relationship to the dosage of leucocytes used and the degree of tuberculin hypersensitivity of the leucocyte donor.

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17243. Acetylcholine-Like Action of a Product Formed by an Acetylating Enzyme System Derived from Brain.*

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In 1943 Nachmansohn and Machado extracted from brain an enzyme, choline acetylase, which forms acetylcholine in cell-free solution using the energy of adenosine triphosphate.[1] The acetylation of choline is a complex reaction requiring for full activity in addition to the enzyme system and ATP, the substrates choline and acetate, a co-enzyme, K^+, Mg^{++}, Ca^{++} ions and cysteine.[2,3] The choline ester formed enzymatically, assumed to be exclusively acetylcholine, was determined by bioassay with the rectus muscle of the frog. In spite of the intense interest of physiologists in acetylcholine, no adequate chemical methods were available and investigators studying the occurrence or formation of the ester used by necessity bioassays, which are of questionable specificity.

Recently high yields of choline ester were obtained by a purified and concentrated enzyme solution which was prepared from acetone dried powder of rabbit brain by fractional ammonium sulphate precipitation. These high yields made possible the determination of choline ester by a chemical method introduced by Hestrin.[4] The method is based on the reaction of O-acyl groups with hydroxylamine in alkaline medium and may therefore be used for the determination of acetylcholine in presence of choline and acetate.

When tested by bioassay the values of acetylcholine formed showed a striking discrepancy with the figures obtained by chemical determination. Less than half of the total biological activity of the enzymatically formed product could be accounted for by the chemical method. Consequently, the greater part of the effect obtained in the bioassay must be attributed to a product which appears to have the same biological action as acetylcholine but may be distinguished from the latter chemically.[3] The formation of a biologically active product is observed in absence of added choline, whereas under these conditions no acetylcholine formation is observed.

It appeared necessary to ascertain whether the product has acetylcholine-like action in other biological systems besides that observed on the frog rectus. Experiments will be described in this paper in which the action of the enzymatically formed product, henceforth referred to as e.f. product, has been tested on the frog heart and on the blood pressure of cats.

The tests of the effect of the product on blood pressure were performed on cats. The animals were anesthetized with Dial (0.07 - 0.08 g/kilo), injected intraperitoneally. Arterial blood pressure was measured in the carotid artery with a mercury manometer and recorded on smoked paper. The compounds tested were injected into the external jugular vein in a volume of 1 ml.

For the experiments on the frog's heart, bullfrogs (*Rana catesbeiana*) were used. The hearts were removed and perfused with frog's Ringer solution according to the method of Straub, as modified by Krayer.[5] Ventricular contractions were recorded with an isotonic lever attached to the apex of the heart.

The enzymatically formed product used in

* This investigation was supported by research grants from the Office of Naval Research and the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

† Rockefeller Foundation Fellow. Present address: Institute of Physiology, University of Chile.

1 Nachmansohn, D., and Machado, A. L., *Neurophysiol.*, 1943, **6**, 397.

2 Nachmansohn, D., and Weiss, M. S., *J. Biol. Chem.*, 1948, **172**, 677.

3 Nachmansohn, D., Hestrin, S., and Voripaieff, H., *J. Biol. Chem.*, 1949, **180**, 249.

4 Hestrin, S., *J. Biol. Chem.*, in press.

5 Krayer, O., Linstead, R. P., and Todd, D., *J. Pharm. and Exp. Therap.*, 1943, **77**, 113.

TABLE I.

Depressor Effect of the Enzymatically Formed Product on Arterial Blood Pressure of Cat. Bioassay with frog's rectus indicated a content of 30-40 μg ACh equivalents per ml of sample. a and b indicate assays done at different periods of the same experiment.

	Acetylcholine		e. f. product		
Exp. No.	μg ACh	Decrease of blood pressure, %	Dilution of product	Decrease of blood pressure, %	μg ACh equivalents/ml
1	0.25	28	400	26	100
2	0.2	22	400	23	80
	0.5	35	200	35	100
3 a.	0.25	25	400	23	100
b.	0.5	30	200	28	100
4	0.25	13	400	12	100
	0.5	35	200	29	<100
5 a.	0.2	17	400	14	80
b.	0.5	26	200	23	100
6 a.	0.2	20	400	24	80
	0.25	36			
b.	0.5	43	200	35	<100
7	0.2	45	400	42	80

all the experiments was obtained by incubation of the choline acetylating system in a reaction mixture as described recently.[3] No choline was added to the mixture. The blank solutions used as controls were aliquot parts of the reaction mixture which formed on incubation the product. The controls were used at O time (without incubation), after the enzyme had been inactivated by short boiling.

Results. A. Action of product on arterial blood pressure of cats. Table I summarizes the results obtained in this series of experiments. In all the experiments performed, the e.f. product exerted on arterial blood pressure a depressor action that closely resembles the fall in blood pressure induced by acetylcholine.

The samples used in Experiments 1 to 3

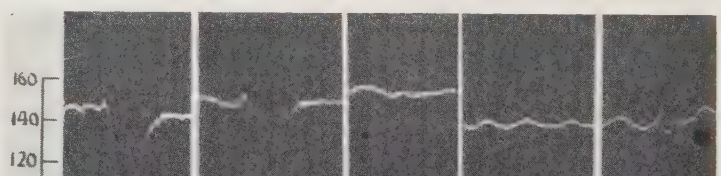

FIG. 1.

Effect of e.f. Product upon Arterial Blood Pressure. Cat. Upper line, arterial blood pressure; second line, injection marks; third line, time in 10 sec. Scale on the left: mm of Hg. 1. ACh 0.5 μg. 2. e.f. product diluted 1:200. 3. Control diluted 1:50. Between 3 and 4, atropine sulfate (0.4 mg). 4 and 5, as in 1 and 2.

TABLE II.

Effect of the Enzymatically Formed Product on the Amplitude of the Frog Heart Contraction. Bioassay with frog rectus indicated a content of 30-40 μg ACh equivalents per ml of sample.

	Acetylcholine		e. f. product		
Exp. No.	μg ACh	Decrease of amplitude, %	Dilution of product	Decrease of amplitude, %	μg ACh equivalents/ml
1	0.01	60	4000	66	50–60
	0.02	78			
2	0.025	58	1600	48	>40
3	0.05	22			
	0.1	40	1600	20	80
4	0.05	32	2000	32	
			1600	40	100
5	0.05	35	1600	37	80
		40	800	50	
6	0.1	50	800	43	
	0.3	64	400	50	40–60
			200	68	
7	0.025	60	1600	68	50–60
	0.05	82			

were derived from the product of the same experiment, 4 to 7 from another experiment. Compared with bioassay on frog rectus the values obtained were higher, although in the same order of magnitude. The figures in all experiments indicate strikingly good constancy. It may be noted that experiments 6 and 7 were carried out about 2 weeks later. During this interval, however, no loss of activity seems to have occurred.

It was also observed that the depressor action of the e.f. product is regularly suppressed by atropine in doses sufficient to abolish the depressor action of acetylcholine.

Fig. 1 shows a typical experiment. It can be observed that a dose of e.f. product (at 1) and of acetylcholine (at 2) which elicit a marked fall of blood pressure before atropine, becomes completely ineffective once the animal has been atropinized (5 and 6). The injection of a control solution at 3 does not modify the level of blood pressure.

B. Action of product on the frog's heart. The results obtained are summarized in Table II. In 7 experiments carried out the e.f. product given in adequate concentrations produced depression of the activity of the heart, similar to that elicited by the administration of acetylcholine. The depressor action affected primarily the amplitude of the cardiac contractions, the frequency being only slightly decreased if at all.

The figures of Table II show that the concentration of the e.f. product in the samples used is fairly constant, as estimated by their action on the frog's heart. It fluctuates between 40 and 80 μg of acetylcholine equivalents per ml in one sample (1 and 2), between 40 and 100 μg per ml in the second sample used (3 to 7).

The cardiodepressor action of the e.f. product was regularly blocked by atropine in concentrations sufficiently high to abolish the cardiodepressor effects of acetylcholine.

The record in Fig. 2 is typical of this series. At 1 and 2, e.f. product and at 3 acetylcholine, produce a marked decrease in the amplitude of cardiac contractions. At 4, a control solution is used; no effect on cardiac activity is obtained. The heart is then atropinized and at 5 and 6, e.f. product and acetylcholine are applied again as in 2 and 3; the

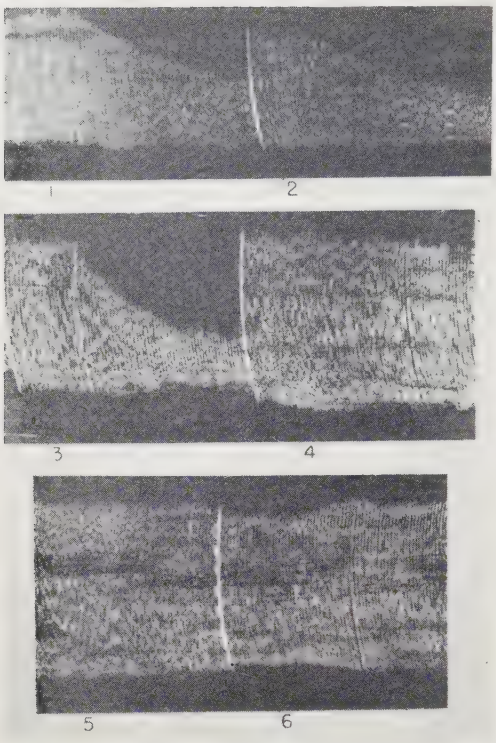

FIG. 2.
Effect of e.f. Product upon the Ventricular Contractions of the Frog Heart.

1. e.f. product diluted 1:100. 2. e.f. product diluted 1:50. 3. ACh 0.3 μg. 4. Control diluted 1:50. 4 and 5. Atropine sulfate 1 mg/liter. 5. ACh as in 3. 6. e.f. product as in 2.

effect on the heart is now abolished.

Summary. The pharmacological properties of an enzymatically formed product described recently by Nachmansohn, Hestrin and Voripaieff have been tested. This product, obtained in the choline acetylating system derived from brain extracts, is distinctly different from acetylcholine but has an acetylcholine-like action in the bioassay with frog rectus.

In the present paper the acetylcholine-like action of the product has been confirmed and extended. The product decreases the arterial blood pressure of cats and the amplitude of the isolated frog heart in the same way as acetylcholine. Atropine regularly suppresses both actions.

We wish to thank Dr. David Nachmansohn for suggesting this research and for his interest and encouragement, and to Dr. S. R. Korey for providing the e.f. product.

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17244. New Substrates for Cholinesterases.

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From the Mayo Foundation, Rochester, Minn.

In previous reports it has been shown that the cholinesterases (ChE) of snake venoms[1-3] and of human erythrocytes[3,4] catalyze the hydrolysis of noncholine esters such as ethyl chloroacetate and β-chloroethyl acetate. Since both enzymes are typical members of the e-group of cholinesterases,[5] the former assumption that the e-ChE was unable to attack noncholine ester, therefore, should be discarded. Thus, a wide field for the search for new substrates of the e-ChE has been opened.

Methods. Esterase activity was measured by the usual manometric procedure.* In each case tests were also run without sub-

† On leave from the University of Basel, Switzerland.

1 Zeller, E. A., *Helvet. physiol. pharmacol. acta,* 1948, **6**, C36.

2 Zeller, E. A., and Utz, D. C., *Helvet. chim. acta,* 1949, **32**, 338.

3 Zeller, E. A., Fleisher, G. A., and McNaughton, R. A., *Fed. Proc.,* 1949, **8**, 268.

4 McNaughton, R. A., and Zeller, E. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 165.

5 Zeller, E. A., *Helvet. chim. acta,* 1949, **32**, 94.

* Twelfth communication: Zeller, E. A., *Helvet. chim. acta,* 1949, **32**, 484.

TABLE I.
Enzymatic Hydrolysis of a Mixture of Acetylcholine and Ethoxyethanol Acetate.*

Substrates	Conc. of substrate	Q	$Q_{ACh} + Q_{EtA}$	$Q_{ACh + EtA}$
ACh	.005 M	5,730	7,490	5,600
EtA	.05 M	1,760		
ACh	.005 M	5,730	8,630	5,080
EtA	.20 M	2,900		
ACh	.01 M	4,700	6,460	4,190
EtA	.05 M	1,760		
ACh	.01 M	4,700	7,600	4,840
EtA	.20 M	2,900		

* 0.1 ml of purified erythrocyte ChE, 1.0 ml total volume.

strates and without enzymes, and all results recorded in this paper were obtained by subtracting the corresponding blanks. They are expressed in terms of Q, which gives the number of microliters of carbon dioxide evolved from bicarbonate-Ringer per hour per milligram of venom or per milliliter of the enzyme solution.

Sources of e-ChE were dry snake venoms (from the family of the Colubridae) and human erythrocytes. The latter were washed 4 times with saline solution and then hemolyzed with 5 volumes of distilled water. The hemolyzed cells were centrifuged at 8,800 g until a reddish precipitate separated. The supernatant was discarded and the precipitate was resuspended in water; this procedure was repeated 6 to 8 times until the supernatant was clear. The orange-red precipitate contained 50 to 60% of the original ChE activity of the erythrocytes. This preparation will be referred to as "purified erythrocyte ChE."

In the venoms[5] and in the purified erythrocyte ChE preparation no indication of the presence of an "ali"-esterase appeared. The activity toward methyl butyrate, which is found in hemolyzed erythrocytes, completely disappeared after purification. Thus, when both preparations attack an ester, it is highly probable that the e-type of ChE is responsible for this reaction. The sensitivity toward eserine and caffeine[6] and, when possible competition experiments with acetylcholine were used to check this conclusion.

Results. A. Ethoxyethanol Acetate (EtA). This glycol derivative is easily attacked by the venoms of *Naia melanoleuca* (Q_{EtA} = 1,720) and *Elaps frontalis* (Q_{EtA} = 170), using a substrate concentration of 0.1 M. Eserine (0.2mM) gives complete inhibition. Purified erythrocyte ChE also catalyzes the hydrolysis of ethoxyethanol acetate. In all cases acetylcholine competed with the noncholine ester, as is seen from the results listed in Table I. The same substance is also hydrolyzed by human serum, a rich source of s-ChE ("pseudo" or "unspecific" ChE). Using a purified erythrocyte ChE and diluted human serum of a similar activity toward acetylcholine (Q_{ACh}), the first enzyme preparation catalyzes the ethoxyethanol acetate much more rapidly than the s-type, in spite of the fact that the affinity between the substrate and the two enzymes is of the same order (compare with Michaelis constant, Fig. 1). Since human serum contains some "ali"-esterase activity[7] which probably is partly responsible for the hydrolysis of the noncholine ester, the ratio might be even more in favor of the e-type.

B. Desoxycorticosterone Acetate (DOCA). The extremely low solubility of this substance in water prevented any rapid reaction. The substance was added to the reaction vessels in dry form. Venom from *Naia melanoleuca* (Q_{ACh} = 30,800)[5] produced an easily readable hydrolysis (Q_{DOCA} = 8.7). Eserine (0.2 mM) inhibited this reaction 90%. Even purified erythrocyte ChE caused a considerable hy-

[6] Zeller, E. A., and Bissegger, Alfred, *Helvet. chim. acta*, 1943, **26**, 1619.

[7] Adams, D. H., and Whittaker, V. P., *Biochem. J.*, 1949, **44**, 62.

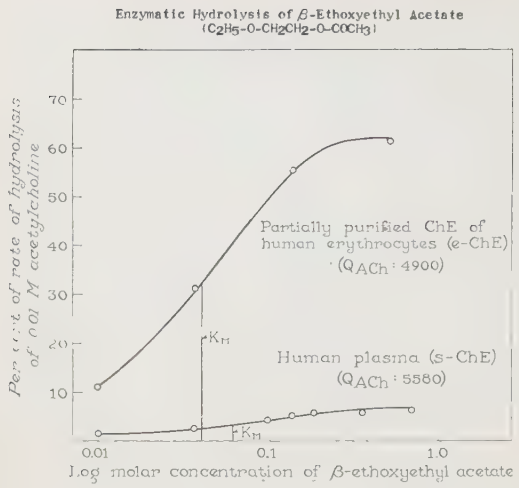

FIG. 1.

Hydrolysis of ethoxyethyl acetate in the presence of human serum and purified erythrocyte cholinesterase; 0.1 ml serum, 0.1 ml purified erythrocyte ChE, total volume 1.0 ml. Reaction velocity expressed in percentage of the reaction velocity caused by 0.01 M acetylcholine. K_M represents the substrate concentration which produced half of the maximal reaction velocity.

drolysis of this ester ($Q_{DOCA} = 48$). On account of the enormous difference between Q_{ACh} and Q_{DOCA} no competitive experiments with a mixture of acetylcholine and DOCA were attempted.

C. Ketopropanol Acetate (KPA). This substance, which, like DOCA, is a keto-alcohol ester, was attacked by the ester of the venom of *Bungarus fasciatus* ($Q_{ACh} = 25{,}000$).[5] At 0.2 M concentration the reaction velocity was $Q_{KPA} = 705$. Eserine (0.2 mM) and caffeine (0.1 M) depressed the rate 100 and 64 per cent respectively.

D. Phenyl Acetates. Phenyl acetate (PA) and various derivatives are hydrolyzed rapidly by preparations of e-ChE and s-ChE (Table II). Like another noncholine ester, ethyl chloroacetate,[4] the hydrolysis of phenyl acetate is inhibited by eserine only in the presence of e-ChE, but not in the presence of human serum. The very rapid hydrolysis of p-nitrophenyl acetate by various snake venoms, by purified erythrocytes ChE and by human serum, and the inhibition of this reaction by the addition of eserine and acetylcholine can easily be followed by the development of the yellow color, due to the liberation of free p-nitrophenol.‡

Comment. Besides acetylcholine the acetates of very different hydroxyl derivatives are hydrolyzed by e-cholinesterase ("true" or "specific" ChE). Acetyl derivatives of unsubstituted aliphatic alcohols and halogenated alcohols have been mentioned previously,[1,3,4] while in the present paper representatives of keto-alcohols, poly-alcohols, steroid alcohols and phenols have been added to the list of the noncholine substrates of e-ChE. On the other hand, much less variation is possible on the part of the acyl group, as has been pointed out by the preliminary results obtained by Bovet Nitti,[8] by Adams and Whittaker[9] and by our laboratory. Of the unsubstituted aliphatic acids acetic acid gives the best results, while acids with more than 3 carbon atoms are not as well hydrolyzed. This behavior separates the e-ChE from the s-ChE ("pseudo"-ChE) and from the "ali"-esterase, because these latter enzymes are active toward esters of acids with more than 3 carbon atoms. At the present time the e-ChE can be considered to be an *acetyl*esterase (e-acetylesterase) rather than a *cholin*esterase.

The ability of the ChE of human erythrocytes to split acetylsalicylic acid and desoxycorticosterone acetate may play a role in the liberation of the corresponding hydroxyl derivatives after the therapeutic application of these and similar substances.

The fact that under certain physiologic and pathologic conditions the ChE activity of a given tissue changed has previously led to far-reaching hypotheses concerning the presence of acetylcholine. Later, similar assumptions were restricted to the e-ChE. In the light of our present results even this latter conclusion can no longer he held. Unless new evidence is produced, no enzymologic clues are available at present to show that acetyl-

‡ The use of phenyl acetate and derivatives thereof for the colorimetric determination of ChE will be discussed elsewhere.

8 Bovet Nitti, F., *Experientia,* 1947, **3**, 283.

9 Adams, D. H., and Whittaker, V. P., *Biochem. J.,* 1948, **43**, xiv.

TABLE II.
Hydrolysis of Phenyl Acetates in the Presence of Various Cholinesterase Preparations.

Source of ChE	Type	Q_{ACh}	Substrate	Q	Q substrate + 0.2 mM eserine
Venom of *Bungarus fasciatus*†	e	25,000	Phenyl acetate, .002 M	16,200	0
Hemolyzed erythrocytes‡	e	11,750	" " .002 M	7,250	0
Plasma*	s	6,500	" " .002 M	117,000	102,000
Purified erythrocyte ChE§	e	12,000	Acetylsalicylic acid, .06 M	160	0
Venom of *Naia melanoleuca*‖	e	30,800	" " .08 M	650	0
Purified erythrocyte ChE¶	e	5,550	*p*-Nitrophenyl acetate, .003 M	2,680	

Total volume in all cases = 1 ml.
* 0.002 ml of heparinized human plasma.
† 0.02 mg of dry venom.
‡ 0.1 ml of washed and hemolyzed erythrocytes; washed 3 times with saline solution and hemolyzed with 3 volumes of distilled water. This suspension was diluted with 9 volumes of "Ringer-30."
§ 0.3 ml (*cf.* "Methods").
‖ 1 mg of dry venom.
¶ 0.025 ml.

choline is the physiologic substrate of the e-ChE.

Summary. e-Cholinesterase ("true" cholinesterase) from human erythrocytes and snake venoms is capable of catalyzing the hydrolysis of ethoxyethanol acetate, desoxycorticosterone acetate, ketopropanol acetate, phenyl acetate, p-nitrophenol acetate and acetylsalicylic acid. The first mentioned ester is attacked more effectively by e-cholinesterase than by s-cholinesterase ("pseudo"-cholinesterase).

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17245. Mobilization of Radioactive Sodium from the Gastrocnemius Muscle of the Dog.*

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The experimental study reported here was based on the clinical application of intramuscular injection of radioactive sodium 24 in studies of peripheral vascular disease by Elkin *et al.*[1] This technic and the inferences drawn from the results of those studies are predicated upon the disappearance of the radioactive sodium by way of the blood stream. However, the possibility existed that a portion of the sodium was being removed by the lymphatic system. A further consideration was that the isotope might diffuse along intramuscular planes, thereby removing itself from the range of the Geiger-Mueller counter, in which event it would erroneously appear that the sodium had been removed by the circulating blood. The study reported here was undertaken, therefore, to determine:

(*1*) The role of the lymphatics in the mobilization and removal of intramuscularly injected radioactive sodium.

(*2*) A means of quantitative estimation of

* This investigation was supported in part by research grants from the U. S. Public Health Service, and the Medical Department of the U. S. Army.

† Research Fellow, Whitehead Department of Surgery.

‡ Electronics Consultant, Whitehead Department of Surgery.

[1] Elkin, D. C., Cooper, F. W., Jr., Rohrer, R. H., Miller, W. B., Jr., Shea, P. C., Jr., and Dennis, E. W., *Surg., Gynec. and Obst.*, 1948, **87**, 1; Cooper, F. W., Jr., Elkin, D. C., Shea, P. C., Jr., and Dennis, E. W., *ibid.*, 1949, **88**, 711.

TABLE I.
Analysis of Chyle for Radioactive Sodium.
Chyle collected from thoracic duct fistulas after injection of isotope into the gastrocnemius muscle.

Dog	Wt, kg	Age of fistula	Chyle collected, cc	Time of collection, hr	Sodium injected, microcuries	% return
1*	11.3	1 day	30	1.5	10	<1
2*	20.0	Immediate postoperative	15	2.5	5†	<1
3	20.0	8 days	30	3	10	<1
4	8.1	1 day	12	3	10	<1
5	10.4	1 day	42	3	15	1.1
6	11.3	1 day post thoracic duct cannulization	16	3.25	13	0.75
7	10.0	1 day	12	1.5	19	<1

* Anesthetized.
† Injection in left hind and forelegs.

radioactive sodium in the blood draining from the injection site.

Role of Lymphatics. The route of egress of any material injected into muscle must be either lymphatic or vascular. Therefore, this portion of the experiment was undertaken to determine if appreciable quantities of the radioactive material could be found in lymph collected from the injected extremity. Although thoracic duct lymph contains lymph from both lower extremities, abdominal viscera, and the left upper extremity, the additional volume and added metabolites found in thoracic duct lymph would not invalidate the accuracy of the counting methods as the presence of the radioactive material could be detected regardless of its dilution. Accordingly, 7 dogs were subjected to operation: thoracic duct fistulas were made in 6, using a modified Biedl technic;[2,3] one additional animal was subjected to thoracic duct cannulation.

Procedure. A longitudinal incision was made along the course of the external jugular vein with ligation and division of all tributaries. The thoracic duct was identified as it entered the external jugular vein approximately at its junction with the left subclavian vein. Identification of the duct was facilitated by feeding the dog one pint of milk and cream 30 minutes prior to operation.

The subclavian and innominate veins were tied and divided below the point of entrance of the thoracic duct, leaving a segment of the external jugular to be used as a conduit to the outside. The vein segment was sutured to the skin, care being taken not to angulate it. A soft rubber catheter was introduced into the fistula and sutured in place; usually the catheter fell away on the first postoperative day.

As it was desirable to collect the chyle from the unanesthetized, ambulatory animal, the following method was devised for this purpose: At the conclusion of the operative procedure, a finger cot, with the end cut off, was sutured around the mouth of the fistula. On the following day, with the animal up and about, another cot was attached by means of Michel clips to the rim of the previously sutured finger cot. This provided a sac into which chyle could drain and which could be changed at periodic intervals.

In one additional dog a polythene cannula was placed in the duct. To protect the cannula, a plaster jacket was placed about the animal's upper thorax, and windows were cut for the cannula and front legs.

Following the animal's recovery from anesthesia, intravenous pentobarbital sodium (Nembutal, Abbott), a 0.1 to 0.2 cc solution of sodium chloride containing 5 to 20 microcuries of radioactive sodium 24 was injected into one of the gastrocnemius muscles. The animal was then encouraged to move about the laboratory, and the thoracic duct lymph was collected for a variable period of time, ranging from 1½ to 3¼ hours. The relative

[2] Biedl and DeCastello, V., *Pfluegers Arch.*, 1901, **86**, 259, cited by Lee, F. C., *Bull. Johns Hopkins Hosp.*, 1922, **33**, 21.

[3] Markowitz, J., Textbook of experimental surgery, William Wood and Co., Baltimore, 1937.

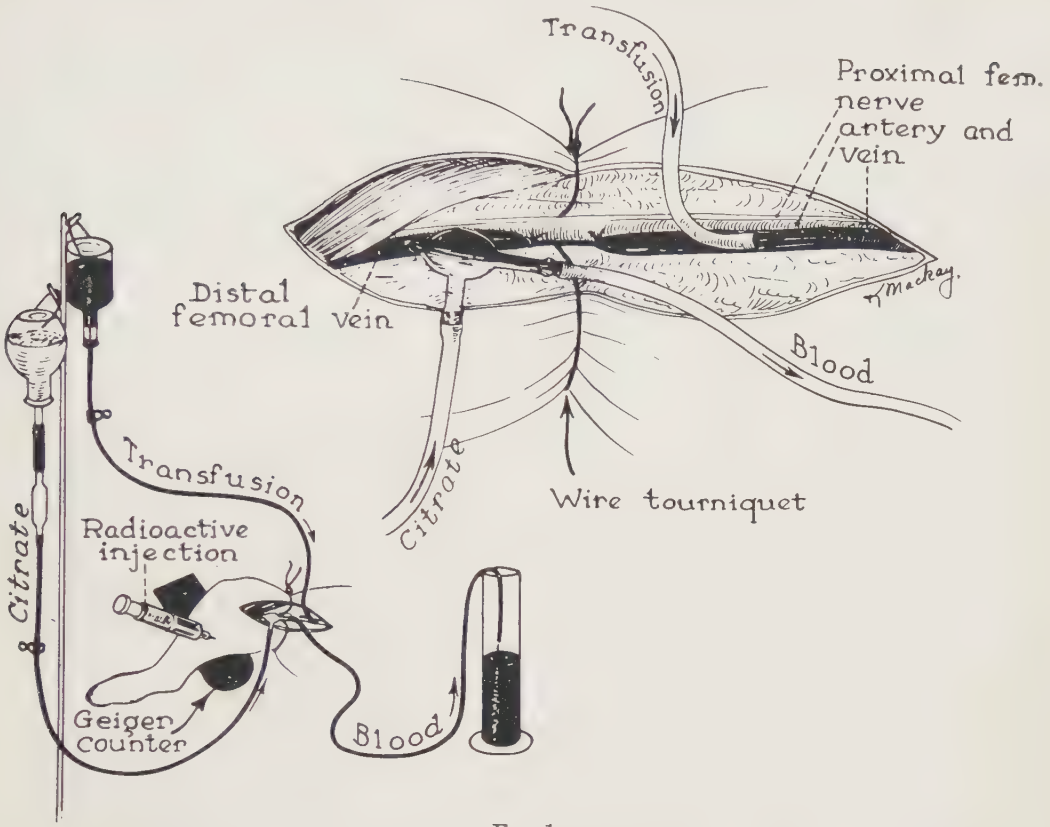

FIG. 1.

a. Geiger-Mueller counter in position to record disappearance of radioactive sodium from site of injection. The femoral nerve, artery and vein are lying above the tightened wire which has compressed and occluded the venous collateral vessels.

b. Relationship of transfusion and collecting cannulas in the femoral vein.

amount of radioactive sodium present in the collected lymph was determined.

As a control, an amount of radioactive sodium equal to that injected was added to a volume of water equal to the volume of chyle collected, and the relative amount of radioactive sodium determined. Correction was made for background radiation, and the relative amounts of sodium in the collected and in the control specimens were compared and expressed in percentage form.

The results of these experiments are recorded in Table I. The 2 anesthetized dogs are included for the purpose of comparing volume of flow in the anesthetized and unanesthetized animals.

Role of the Capillary and Venous Systems. The radioactive sodium content of blood returning from the gastrocnemius muscle was studied in 9 dogs. In 2, the procedure was repeated in the opposite leg after an interval of at least 3 days. Therefore, a total of 11 studies was made.

Procedure. With the animal under intravenous pentobarbital sodium anesthesia, a longitudinal incision was made over the femoral nerve, artery and vein. These structures were isolated, and a malleable copper wire placed beneath them and brought around the thigh so as to completely encircle it. On tightening the wire, all vessels of the leg, with the exception of the femoral, were compressed; therefore, any sodium being mobilized at the capillary loop and returning to the general circulation had to pass cephalad via the femoral vein.

A Geiger-Mueller counter connected to a

recording mechanism§ was placed under the leg, just beneath the gastrocnemius muscle. A Jackson cannula was introduced into the distal segment of the femoral vein, and sodium citrate, 2.5%, was allowed to flow in one arm of the cannula and mix with the blood as it left the vein. Attached to the third arm of the cannula was a rubber tube which ran to a collecting flask outside the operative field. To obviate shock, a condition incompatible with normal sodium mobilization, blood from healthy mongrel dogs was given to maintain blood volume. The transfusion technic was to introduce a glass cannula into the proximal end of the femoral vein and replace the blood by way of this cannula. The transfusion was regulated so that blood was replaced at the same rate it was withdrawn. At the moment the collecting cannula was introduced, a saline solution containing 7 to17 microcuries of radioactive sodium was injected into the gastrocnemius muscle directly over the Geiger-Mueller counter. Fig. 1 illustrates the equipment and methods.

The blood was collected for periods ranging from 10 to 20 minutes. During this time the amount of radioactive sodium present at the

§ Wire recorder.[4] To simplify the procedure at the time of the experiment and to minimize the factor of human error inherent in recording the counting rate by the usual method, an electromechanical system was used in which the impulses from the Geiger-Mueller counter were recorded as they occurred, and analyzed at a later time under more favorable conditions (*i.e.*, in the isotope laboratory rather than in the animal operating room).

A portable wire recorder was used, into which the output of the Geiger-Mueller counter was fed after being passed through an appropriate amplifier. The result was a complete record of the counts as they occurred at the site of injection for the entire period of the phlebotomy.

The wire recording was then fed through an amplifier and conventional scaler, and thence to a translator which drove a mechanical chart recorder. The chart produced was a complete record of the impulses in their order and time of occurrence, from which the counting rate for each minute throughout the run could be computed and plotted.

[4] Kety, S. S., *Am. J. Med. Sciences*, 1948, **215**, 352.

TABLE II.

Analysis of Femoral Vein Blood for Radioactive Sodium. Isotope injected into the gastrocnemius muscle and wire tourniquet tightened.

Dog	Wt, kg	Estimated* blood volume, cc	Blood collected, cc	Duration of experiment, min.	Sodium injected, microcuries	% removed (muscle)	% collected (blood)	"K" value (muscle)	"K" value (blood)	% deviation
8	15.9	1462	1092	15	11.5	54	49	0.051	0.045	— 9
9	15.9	1462	750	15	16.5	47	43	0.042	0.038	— 9
10	8.1	745	250	15	11.4	42	17	0.036	0.012	—60
11	7.7	708	640	20	7.6	52	51	0.037	0.036	— 2
12	14.0	1288	750	13	16.5	45	46	0.046	0.047	+ 2
13	12.7	1168	490	18	10.5	80	78	0.089	0.083	— 1
14	14.5	1334								
Right leg			615	15	9.4	47	46	0.042	0.041	— 2
Left leg			385	15	12.6	60	67	0.061	0.073	+12
15	7.2	662								
Right leg			425	16	8.0	60	65	0.057	0.065	+ 8
Left leg			400	15	12.9	62	61	0.064	0.063	— 2
16	10.0	920	260	15	12.8	50	48	0.046	0.043	— 4

* Blood volume was estimated on the basis of 92 cc/kg of body weight.

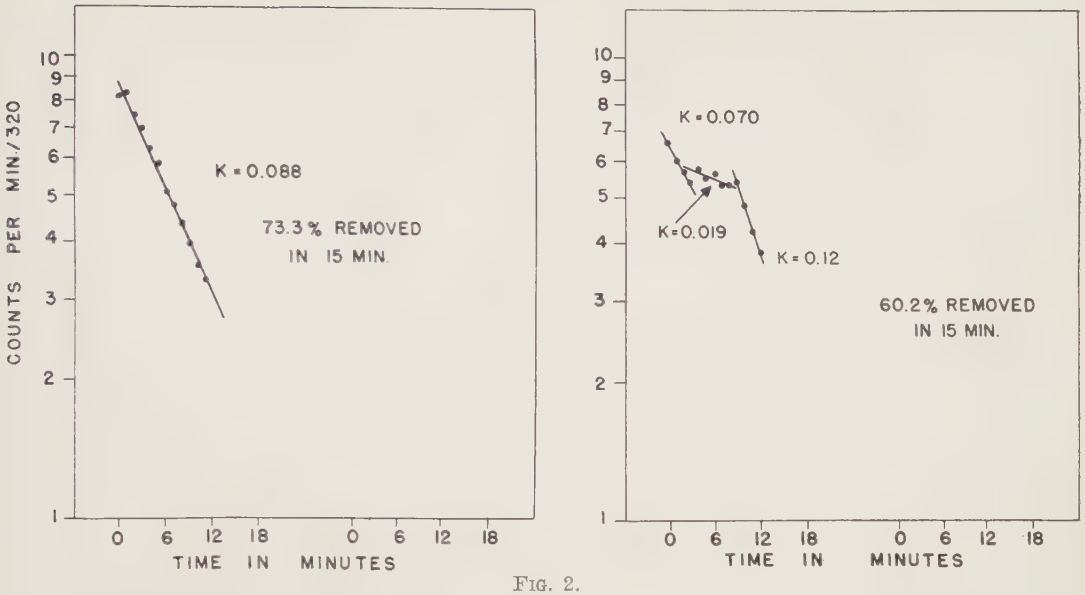

FIG. 2.

Graphs showing rates of mobilization of radioactive sodium from the gastrocnemius muscle of dogs; *a*, uniform rate; *b*, fluctuating rate. The line representing the rate of removal is obtained by plotting the counting rate for each minute against time.

site of injection was recorded continuously.

The radioactive content of the collected blood was determined by the same method used for the thoracic duct lymph. The relative amount of radioactive sodium in the collected blood was compared with a control sample also prepared by the same method used for lymph control. The amount of radio active sodium collected was expressed as a percentage of the amount injected, or, more simply, percentage return in the blood.

The rate of mobilization of the radioactive material from the muscle was determined from the collected blood by the equation:[4]

$$K_1 = \frac{2 - \log(100 - P)}{.4343\ T}$$

K_1 = Tissue clearance constant as determined from the collected blood.
T = Time elapsed or duration of phlebotomy.
P = Per cent of sodium recovered in the collected blood.

The rate of mobilization was also determined directly at the site of injection by plotting against time on semilogarithmic paper the number of counts for each minute throughout the phlebotomy, as detected by the Geiger-Mueller counter at the injection site. Mathematically, this rate may be expressed by the equation:

$$K_2 = \frac{\log C_2 - \log C_1}{.4343\ (T_2 - T_1)}$$

K_2 = Tissue clearance constant as determined by measurement directly at the site of injection.
C_1 = Number of counts at beginning of phlebotomy or Time T_1.
C_2 = Number of counts at end of phlebotomy or time T_2.

The results of 11 procedures are shown in Table II. Comparison can be made of the rates of removal (K values), percentage removed (as measured at the muscle) and percentage returned (as measured from the blood) along with their percentile deviation.

In some instances there was variation in the rate of sodium removal from the site of injection, necessitating multiple determinations of K value and percentage removed at this site (muscle). Fig. 2 illustrates both a constant and sporadic or irregular rate of mobilization.

Results. The lymphatic system of the dog's leg did not play a significant role in the mobilization and removal of the intramuscularly injected radioactive sodium. In no instance was the quantity of radioactive sodium recovered in the thoracic duct lymph greater than 1.1% of the amount injected into the gastrocnemius muscle.

The data obtained on the group of dogs in which the collateral venous system of the thigh was occluded by a wire tourniquet (Table II) shows good correlation between the percentage known to have been mobilized and the percentage actually recovered in the blood draining the limb.‖

Failure to recover 60% of the isotope in the case of dog No. 10 may be attributed to one or both of the following factors: It is possible that the wire was not tightened sufficiently to occlude completely the collateral channels; it is possible that a thrombus might have partially occluded the lumen of the cannula. A thrombus would prevent a free flow of blood, resulting in stasis, dilatation of the femoral vein and tributaries, and increased venous pressure. The volume of blood collected in this dog was low, a finding which tended to substantiate the latter hypothesis.

The remaining 10 analyses in this series showed percentage deviations within 12%. Control studies showed that a 12% error was possible with this technic.¶ Therefore, it was concluded that radioactive sodium could be successfully recovered from the blood draining the injection site and that the mobilization of this material was solely a function of the capillary loop. In view of the fact that the isotope was not found in the thoracic duct lymph, and that it was recovered quantitatively in the blood, the factors of lymphatic removal and diffusion beyond the range of the Geiger-Mueller counter appeared to be insignificant. The assumption that removal of sodium 24 was a function of the hemovascular system, and consequently, was dependent upon the state of the circulation at the site of injection, was apparently valid.

Summary. A study of sodium mobilization from the gastrocnemius muscle of dogs by the use of radioactive sodium 24 is reported.

The sodium content of thoracic duct lymph following injection of sodium 24 into the gastrocnemius muscle was measured in 7 dogs to determine the role of the lymphatic system in the mobilization of the sodium.

The capillary mobilization and venous transport of sodium 24 from the gastrocnemius muscle was studied in 11 extremities. The radioactive sodium content of the blood draining from the gastrocnemius muscle was determined by two methods: (*1*) directly, by actual measurement of the sodium 24 content of collected blood, and (*2*) indirectly, by means of a Geiger-Mueller counter placed beneath the site of injection of the radioactive sodium into the muscle.

Conclusions. The lymphatic system of the dog's hind extremity does not play a significant role in the mobilization of intramuscularly injected sodium, nor does the radioactive material diffuse along intramuscular planes beyond the range of the Geiger-Mueller counter.

Intramuscularly injected radioactive sodium can be quantitatively recovered from femoral vein blood if the collateral venous system of the upper thigh is occluded.

The rate of removal of sodium 24 measured external to the site of injection is the same as that determined from the blood actually draining from the muscle.

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‖ The radioactive sodium content of femoral vein blood in the absence of collateral venous occlusion was variable. Ten dogs were subjected to femoral phlebotomy without the application of a wire tourniquet, and the radioactive sodium content of the femoral vein blood determined after injection of the sodium into the gastrocnemius muscle. It was possible to recover the total amount known to have been removed from the muscle in only 5 animals; in the remaining 5, a portion of the sodium mobilized, ranging from 15 to 60%, was shunted through venous collateral channels.

¶ To determine the percentage of error inherent in the counting technic used in this experiment, equal quantities of sodium 24 in the form of sodium chloride were placed in equal volumes of water and blood, and counting rates determined. It was found that the percentage of error in counting rates between the two fluids varied from 2 to 12%. Accordingly, the results have been interpreted in the light of a possible 12% deviation.

17246. Is the Salivary Lactobacillus Count a Valid Index of Activity of Dental Caries?*

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(Introduced by P. C. Jeans.)

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Lactobacillus counts are used widely as an index to the activity of tooth decay. Much of the current philosophy relating to the cause and control of dental caries is based on the premise that the Lactobacillus count of the saliva provides a dependable prediction medium as to whether the individual in question is likely to develop tooth decay within the reasonably near future. Yet few studies have been reported wherein Lactobacillus counts have been correlated individually with the previous and subsequent progress of tooth decay over long periods of time for large numbers of subjects. Studies of that type are needed to show the validity of the Lactobacillus count as a diagnostic or prognostic agent. The study reported herewith was designed to supply evidence as to the significance of the Lactobacillus count to the individual subject.

As a part of a larger and more general survey, the Lactobacillus counts from the saliva of 64 teen-aged girls have been compared with the progression of tooth decay observed for not less than 30 months for each individual subject. The data include 407 separate Lactobacillus counts. The dental examinations were made recurrently from July, 1946 to January, 1949; the Lactobacillus counts were made on successive occasions from March, 1948 until January, 1949. The State Hygienic Laboratories made the bacterial counts, as a part of the service they provide to the dentists of the state for clinical diagnosis and prognosis.

Generally speaking, there was a slight trend toward parallelism of the Lactobacillus counts and rates of progression of caries when massed data were used. However, when the group as a whole was subdivided according to the rate of caries progression, there was little difference between the range of Lactobacillus

TABLE I.
Lactobacillus Counts from 64 Subjects Observed Serially for 2½ Years.

	(a) those with no advance of caries	(b) those with greatest advance	(c) total group
No. of subjects	11	15	64
No. of counts	68	105	407
Mean number of Lactobacilli per cc	92,500	112,000	104,500
S.D.	70,000	106,300	88,500
S.E. mean	8,500	10,400	4,040
Decile rating:			
10th	30,000	35,000	30.000
20th	37,000	48,000	43,000
30th	46,000	60,000	50,000
40th	60,000	65,000	60,000
50th	70,000	80,000	72,000
60th	92,000	96,000	90,000
70th	100,000	125,000	120,000
80th	149,000	150,000	144,000
90th	180,000	200,000	180,000
100th	360,000	720,000	720,000
Avg. No. of new DMF tooth surfaces per annum	None	more than 3.5	2.12

* This study was made possible through a grant from the Sugar Research Foundation.

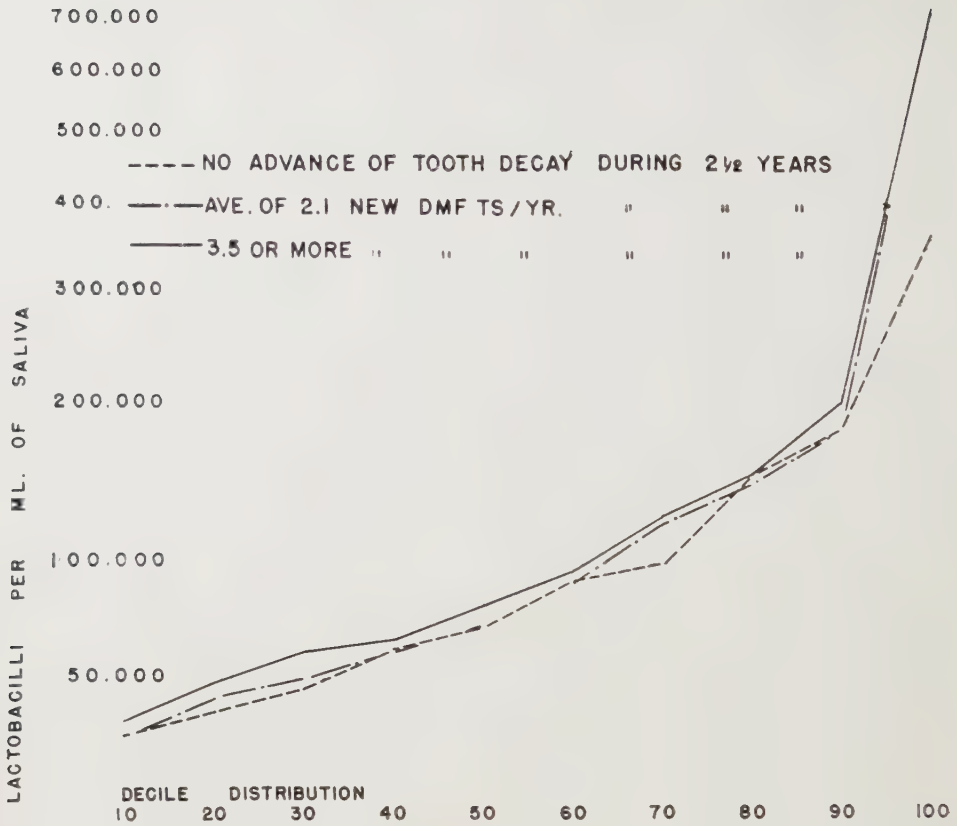

FIG. 1.

Decile range for Lactobacillus counts observed from saliva of teen-aged girls whose rates of progression of tooth decay (number of newly affected tooth surfaces: decayed, missing or filled: per annum) were observed individually for not less than 30 months. Separate curves are shown for 11 girls with zero progression, for the 15 girls with the most rapid progression, and for the total group of 64 subjects. The hundredth per centile represents the highest value observed for each sub-group.

counts observed among those with the least and those with the greatest progression of tooth decay.

Eleven of the girls showed no measurable progression of tooth decay throughout the period of more than 2½ years, when scored according to the number of new decayed, missing or filled (DMF) surfaces which developed during the interim. The range of the Lactobacillus counts observed from these girls' saliva samples has been contrasted with the range for the entire group of 64 subjects, and also with the range observed among the 15 girls whose caries progression rates were the highest. The results are summarized in Table I and Fig. 1.

Emphasis is directed toward the high Lactobacillus counts observed among those children who had had no progression of tooth decay during the 20 months which preceded their first Lactobacillus count, and who continued free from evidence of caries advance at least for the 10 months which followed. Obviously the persistence of high Lactobacillus counts in these children's mouths had not led to the initiation of tooth decay during the period of observation. Moreover, for these 11 girls out of a group of 64 subjects the Lactobacillus count failed to serve as a proper index of caries susceptibility. Furthermore, analysis of the remainder of the group shows such inconstancy of relationship between the magni-

tude of the Lactobacillus count and the relative rate of caries progression that one cannot consider the test as definitive within this group for the diagnosis or the prognosis of caries activity for the individual subject.

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17247. Nucleoproteins and the Cytological Chemistry of *Paramecium* Nuclei.*

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There are many exceptions to the classical definition of a cell as "a mass of protoplasm containing a nucleus."[1] Numerous examples are known of binucleate and multinucleate protoplasmic masses. In most cases the nuclei are all alike, but in binucleate ciliated protozoa such as *Paramecium*, the two nuclei are markedly different in size, and are therefore known as the macronucleus and the micronucleus. There are also considerable differences in behavior of these two nuclei. It is the general view that the macronucleus is a "somatic nucleus" serving the indispensable physiological needs of the organism in vegetative stages when no detectable effect of the micronucleus can be observed.[2] The micronucleus appears to be of chief importance as a potentially germinal structure, functioning in the nuclear interchange and reorganization which take place at conjugation or autogamy.[2] The present paper briefly reports the results of a study of the nucleoprotein composition of macro- and micronuclei in vegetative individuals of *Paramecium*; experiments were designed to answer the question whether in these stages the two nuclei are as unlike as other experimental evidence indicates them to be.

Experimental. The chemical constituents of nuclei are mainly nucleoproteins, and the quantitative technics of cytological chemistry are particularly suited to analysis and localization of these substances within individual nuclei. The procedures used in this investigation permit the computation of relative concentrations of nucleic acids and proteins from specific chemical reactions which have been shown to be applicable to properly fixed cytological preparations. Analyses were made by microscopic photometry of total and nonhistone protein from modifications of the Millon reaction,[3,4] of desoxyribose nucleic acid (DNA) from the Feulgen nucleal reaction,[5] of total nucleic acids and polynucleotides (removable with hot trichloroacetic acid) from the absorption in the ultraviolet (near 260 mμ) of their purine and pyrimidine bases[6,3] and of ribose nucleic acid (RNA) by difference in absorption at 260 mμ after enzymatic digestion with protease-free ribonuclease.[7]

Determinations were made on a culture of *Paramecium caudatum* which was raised on a baked lettuce medium inoculated with *A. aerogenes*[8] and kept under constant feeding

* Part of the research reported here was carried out at Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

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TABLE I.
Comparison of Nucleic Acid and Protein Contents of the Macro- and Micronucleus of *Paramecium caudatum*.

	Total protein	Desoxyribose nucleic acid	Ribose nucleic acid
A. % macronuclear nucleoprotein determined	85.1	5.8	9.1
B. % micronuclear nucleoprotein determined	87.6	4.2	8.2
C. Ratio concentrations: Macronucleus / Micronucleus	0.9	1.2	1.0

and environmental conditions. At no time during the life of the culture was autogamy or conjugation observed; all individuals were in a "vegetative" state. The culture was concentrated by mild centrifugation, fixed in Carnoy's 1:3 acetic acid-alcohol, transferred to small agar blocks and imbedded in paraffin. Analyses were carried out on serial sections mounted on slides in the usual manner.

Mean extinction values ($\log_{10} I_0/I$) were computed from transmission measurements of cylindrical areas of a large number (30-50) of random sections of macronuclei, and of 8-12 entire micronuclei. The results were fairly uniform, as shown by the fact that the standard errors in no case exceeded 7% of the mean extinctions. From these mean values concentrations were computed by reference to standards measured on known dilute solutions in a Beckman Spectrophotometer. An approximation to the protein concentration was computed by assuming that the Millon reaction was due to a tyrosine derivative which constituted one-sixteenth (6.25%) of the protein. The total nucleoprotein concentration was computed by adding together the mean concentrations of protein, desoxyribose nucleic acid (DNA), and ribose nucleic acid (RNA). Table I (A and B) shows the percentages of the total concentration represented by each of these components.

Results. It is apparent from the data that protein is the major component of the nucleus (Table I), comprising over 85% of the nucleoprotein determined for each nucleus. A comparison of the extinctions of nuclei prepared to show total and non-histone proteins showed no significant difference and it followed that histone could not have comprised more than a few percent of the nucleoprotein. The protein/DNA ratio was from 15-20 to 1, in the range of high values reported by Pollister and Leuchtenberger[4] for metabolic nuclei of metazoa. The concentration of DNA present in the nuclei was quite comparable with determinations of other workers, being in the order of 10^{-12} mg per cubic micron. Most remarkable, however, are the results of digestion with purified crystalline ribonuclease[9] which show that about 60% of the total nucleotide extinction was lost after treatment and that a large quantity of RNA was consequently present. The RNA released by the enzyme amounted to almost twice the DNA present in the nucleus and comprised about 10% of the nucleoprotein moiety. That RNA might be contained in the macronucleus of *Paramecium* has already been indicated by the staining experiments of Shubnikova[10] and others who, with methyl green and pyronin (unpurified), showed pyronin coloring in the macronucleus removable with ribonuclease (unpurified). RNA is known to be a component of metazoan nuclei both in the nucleolus and in the chromosomes. Mirsky has indicated by his analyses of isolated mammalian chromosomes that the RNA which is a component of the residual chromosome, is high in active metabolizing cells and low in relatively inactive ones.[11]

Discussion. It can be seen from the ratios

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[10] Shubnikova, E., *C. R. Acad. Sci. URSS*, 1947, **55**, 517.

of concentrations in Table I, row C, that the macro- and micronuclei are very much alike in nucleoprotein composition. It follows, then, that insofar as their chemical composition is indicative of their function, both nuclei are performing similar roles in the vegetative individual. Thus, the organism's dependence on the macronucleus and its apparent independence of the micronucleus during these stages[2] simply becomes a matter of the preponderance of the physiological-genetic material in the large macronucleus. There is a marked resemblance between these protozoan nuclei and highly metabolic nuclei of metazoa, as typified by the mammalian liver nucleus, which is strong evidence for the active participation of both macro- and micronucleus in metabolic functions of the cell. Any chemical differences which may serve to be correlated with the capacity of the micronucleus to differentiate into a special reproductive structure must be looked for at some other time in the organism's history. Presumably this is during the reproductive processes of conjugation and autogamy, when the behavior and morphology of the two nuclei are most markedly dissimilar.

Summary. Quantitative cytological chemical analyses of the nucleoprotein composition of the macro- and micronuclei of a clone of *P. caudatum* show that both nuclei contain protein, RNA and DNA in the ratio of 20:2:1. Twice as much RNA as DNA was found. From their resemblances to the nucleoprotein contents of metabolic metazoan nuclei, it is concluded that in the vegetative stage, both nuclei of *P. caudatum* are actively metabolic.

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17248. Influence of Level of Adrenal Cortical Steroids on Sensitivity of Mice to X Irradiation.

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We reported previously that X radiation appeared to result in an increased demand for the adrenal cortical hormone.[1] Subsequent studies indicated that the adrenal changes noted after total-body X irradiation of rats (decreased cholesterol content and increased weight) were mediated by the pituitary.[2] The adrenal response could be prevented, in part, by suitable administration of adrenal cortical extract. However, such treatment failed to alter survival.[3] Since Ellinger[4] reported earlier that the daily injection of desoxycorticosterone increased survival of irradiated mice, it seemed desirable to extend these observations in order to clarify the role of the adrenal in the acute radiation syndrome. In these experiments we have determined the radiosensitivity of intact mice with and without whole adrenal cortical extract or desoxycorticosterone. Data on the sensitivity of adrenalectomized mice which were obtained as part of another study have been included for comparison.

Materials and Methods. Male and female CF1 mice, weighing 18 to 28 g each, were the experimental animals. Mice of the same sex were used in individual experiments. For the irradiation, the animals were placed in individual cellulose acetate exposure cells and were given total-body exposures in groups of 16. The cells were rotated slowly on an electrically-driven turntable to insure equivalent irradiation of all the animals. Equal numbers of control and experimental mice

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[2] Patt, H. M., Swift, M. N., Tyree, E. B., and Straube, R. L., *Science*, 1948, **108**, 475.

[3] Swift, M. N., Patt, H. M., and Tyree, E. B., *Fed. Proc.*, 1948, **7**, 121.

[4] Ellinger, F., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 31.

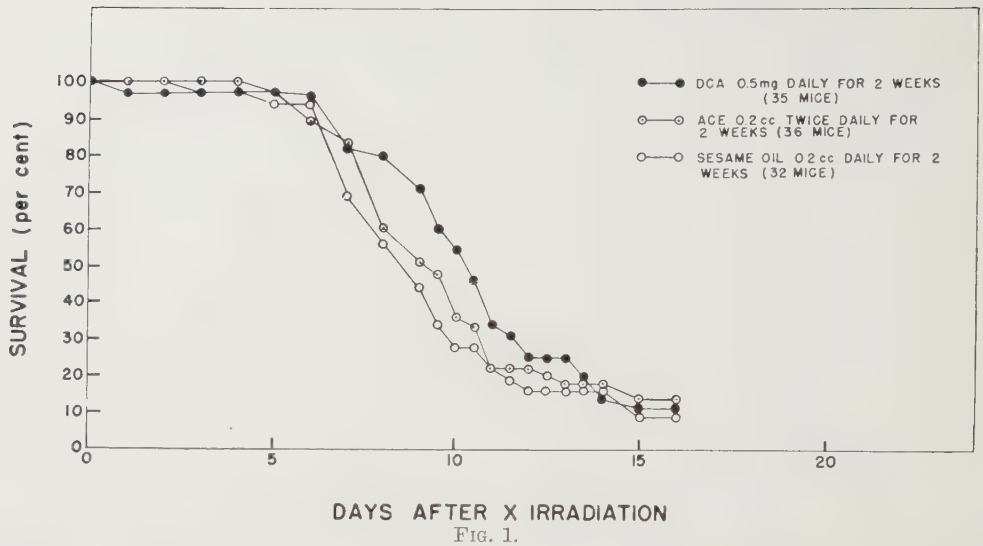

FIG. 1.

Effect of desoxycorticosterone (DCA) and whole adrenal cortical extract (ACE) on radiation mortality in mice (CF1 males, 500 r).

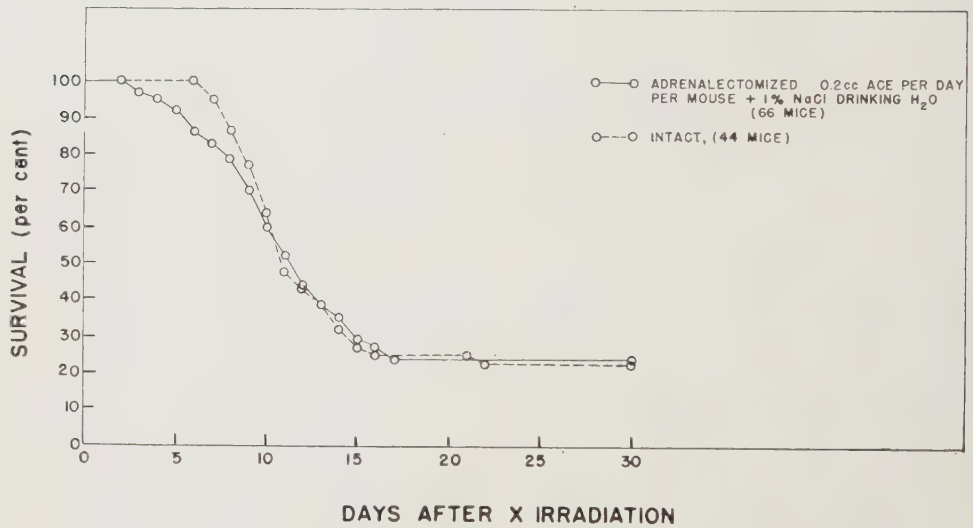

FIG. 2.

Effect of whole adrenal cortical extract (ACE) on radiation mortality in adrenalectomized mice (CF1 males, 500 r).

were used for each exposure. The radiation factors were: 200 kv, 15 ma, 0.5-mm Cu and 3.0-mm Bakelite filters, HVL-1.20-mm Cu, target distance, 10.8 cm, and dose rate, 20 r per minute. Male mice received 500 r and females, 550 r (measured in air).

Single stage bilateral adrenalectomies were performed under nembutal anesthesia 15 to 17 days prior to irradiation. All animals received a postoperative injection of 0.2 cc whole adrenal cortical extract (Wilson).

Survival of irradiated mice was compared under the following 4 experimental conditions:

1) Mice injected intramuscularly with desoxycorticosterone acetate (Schering, 0.5 mg daily) for 2 weeks after irradiation. The irradiated control group received equivalent amounts of sesame oil.

2) Mice injected subcutaneously with whole

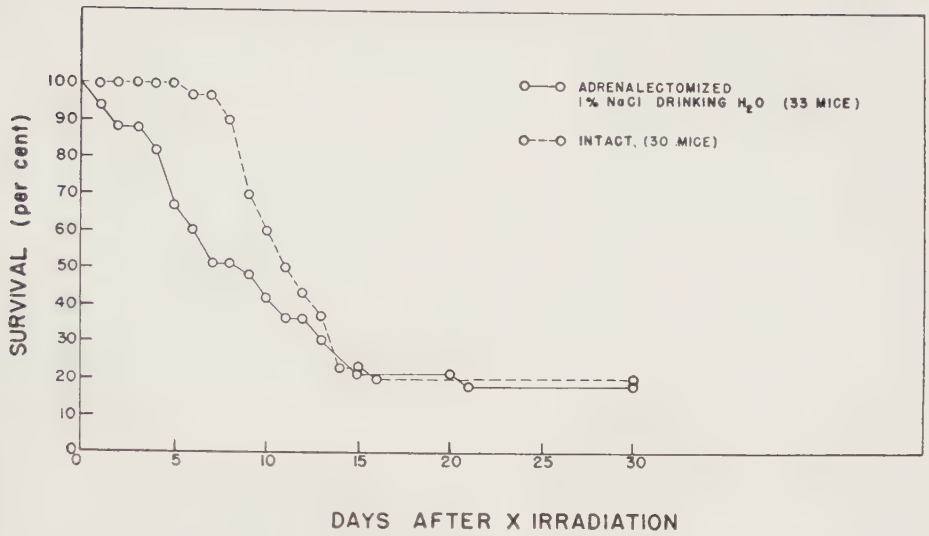

FIG. 3.
Effect of adrenalectomy on radiation mortality in mice (CF1 females, 550 r).

adrenal cortical extract, 0.2 cc, twice daily, for 2 weeks following X irradiation. The control group in this instance was identical with that of 1) above.

3) Bilaterally adrenalectomized mice maintained on salted drinking water (1 per cent NaCl) and daily injections of 0.2 cc whole adrenal cortical extract. Intact irradiated mice were used as controls.

4) Bilaterally adrenalectomized mice maintained on salted drinking water alone, plus an intact irradiated control group.

Results and Discussion. As may be noted in Fig. 1 and 2, mice that received desoxycorticosterone acetate or whole adrenal cortical extract, as well as those that were adrenalectomized and maintained on whole adrenal cortical extract plus salt exhibited a final radiation mortality identical with that of intact irradiated controls. Although there was a decreased mean survival time ($p << 0.001$), those adrenalectomized animals maintained on salted drinking water alone (Fig. 3) also show a final mortality similar to that of the normal irradiated controls and the 3 experimental groups noted above.

The schedule of treatment used in administering desoxycorticosterone is that reported by Ellinger to yield maximal protection in terms of survival.[4] We are unable to account for the failure of desoxycorticosterone to modify survival in our studies. This disparity in results may reside in the different strain of mice used. It is of interest to note that both larger and smaller doses of desoxycorticosterone were ineffectual according to Ellinger.

From these data we may conclude that the survival of X-irradiated mice appears to be independent, at least within certain limits, of the amount of adrenal cortical steroids present. The adrenals are apparently involved only secondarily as part of the organism's buffer response to the stress of irradiation. The rationale of employing adrenal corticoids to alter radiation mortality is therefore open to question.

Summary. The experiments cited indicate that the radiosensitivity of intact or adrenalectomized mice, with or without exogenous adrenal cortical steroids, is similar.

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17249. Reactions of Normal Ovaries to Injections of Stilbestrol.*

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It is a common belief that injected hormones do not stimulate the organs which normally produce them. While injected estrogens may have no direct stimulatory effect on ovarian follicles of normal adult animals, they do produce certain ovarian changes either by direct action on the ovaries, or indirectly via the anterior pituitary gland.[1,2] The consensus is that, if the injected doses are of sufficient potency, there will be (1) luteinization,[3-5] and (2) inhibition or depression of follicular development,[2,6,7] and that these effects are secondary to pituitary changes produced by increased estrogen. It is probable that FSH normally stimulates medium-sized follicles to develop fully to the ovulating stage. If the estrogen level goes over a certain point, the production of FSH is inhibited or depressed, thus resulting in failure of the development of ovulating follicles. At the same time, LH is liberated, either as a result of inhibition of FSH or due to the direct stimulatory effect of increased estrogen, and LH stimulates luteal formation in the ovary. There is the possibility that luteinization is an effect of increased estrogens acting directly on the ovaries, but assays of pituitary glands after estrogenic treatment show an increased potency of luteinizing function.[8]

Materials and methods. Follicular development and luteinization in the ovaries of normal adult rats were studied after intraperitoneal injections of large doses of the synthetic estrogen, diethylstilbestrol dipropionate ("Estrobene DP"). Inbred Sprague-Dawley rats were used, and the work was done on 100-day-old virgin females. The ovaries of 11 control rats were removed at the 5 stages of the estrous cycle and were serially sectioned. In the experimental group, consisting of 15 rats, 5 mg of stilbestrol were injected intraperitoneally once per day, ranging from 1 to 12 injections. All of the injections were begun at diestrus. The ovaries were removed 24 hours after the last scheduled injection, were fixed and serially sectioned. From the serial sections of the control and experimental groups the following determinations were made: (1) the total volumes of the ovaries were determined by planimeter measurements of serial projection drawings; (2) the total volumes of the luteal tissue were measured by the same methods, and the percentage luteinization determined in each case; (3) the follicles were counted and divided into 4 categories: normal and atretic small follicles, and normal and atretic follicles in which an antrum had definitely formed.

Results. In the results, which are summarized in Table I, the animals are divided into 4 groups, and the average figures are given for each group. Group A consists of the 11 control animals in the various stages of the normal 4-day estrous cycle. Groups B, C, and D consist of 15 animals which received daily injections of 5 mg of diethylstilbestrol over a period ranging from 1 to 12 days. In Group B, the animals received 5 to 20 mg in the 4-day period ranging from 1 to 4 days; in Group C, 25 to 40 mg in the

* This work was aided by the fund contributed by the M. D. Anderson Foundation. The author wishes to express his appreciation for the excellent technical assistance of Miss Sarah Lea O'Neill.

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TABLE I.
Summary of Effects of Stilbestrol on the Ovary.

Determinations	Group A, control, avg	Group B, 1-4 day stilbestrol, avg	Group C, 5-8 day stilbestrol, avg	Group D, 9-12 day stilbestrol, avg
a. Volume of ovaries in 1000 planimeter units	46.6	39.8	44.9	56.9
b. Volume of corpora lutea in 1000 planimeter units	20.7	16.1	21.2	30.4
c. % luteinization	44.2	40.1	45.8	53.0
d. Total No. of follicles	435	292	235	281
e. Total No. of normal follicles	334	229	187	190
f. Total No. of atretic follicles	101	62	48	91
g. No. of normal small follicles	259	159	97	75
h. No. of atretic small follicles	61	36	18	24
i. No. of normal antrum-containing follicles	75	70	80	115
j. No. of atretic antrum-containing follicles	40	26	30	67

4-day period ranging from 5 to 8 days; and in Group D, 45 to 60 mg in the 4-day period ranging from 9 to 12 days.

With progressive injections of stilbestrol there is an initial decrease in ovarian size during the first 6 days, followed by a marked increase over the normal size (Table I, a). Similarly with luteal tissue (Table I, b), there is an initial decrease followed by a marked increase in luteinization, which is directly proportional to the increase in ovarian size. The degree of luteinization is shown in Table I, c. During the stilbestrol injections there is an initial 4-day lag followed by a marked increase in the degree of luteinization. The initial lag may be due to a natural hypophyseal-gonadal lag, which has been indicated by the 2-4-day delay in the effect of hypophysectomy on ovaries.[9]

Following treatment with stilbestrol there is a significant decrease in the total number of follicles from the second day on (Table I, d). This consists of a decrease in the number of both atretic and normal follicles (Table I, e,f), with some recovery in the number of atretic follicles towards the end of the experimental period. If there is a complete physiological block of FSH after injections of massive doses of stilbestrol, one would expect the follicular picture to resemble that after hypophysectomy, except for any direct effects stilbestrol may have on the ovaries. Smith[9] has described the follicular picture after hypophysectomy in the rat as follows: (1) all medium-sized and large follicles (*i.e.*, all antrum-containing follicles) become atretic within 4 days or less, and formation of normal follicles in this group is terminated; (2) small follicles continue to develop, but become atretic before antrum formation. In an attempt to analyse the effects of large doses of stilbestrol, the follicles in these two categories were counted and the number of normal and atretic follicles determined in each.

It is evident that there is a continuous and rapid decline in number of normal small follicles with progressive stilbestrol injections (Table I, g). Similarly, as shown in Table I,h, there is a decrease in the number of atretic small follicles. This decrease in the number of small follicles indicates that in our series

[9] Smith, Philip E., *Am. J. Anat.*, 1930, **45**, 205.

there is an inhibition or depression of the formation of small follicles which is a direct effect of stilbestrol on the ovary. Table I, i shows that, after a slight initial depression during the first 4 days of injection, there is an increase in the number of normal antrum-containing follicles. This increase indicates that follicular development still proceeds from the small to the antrum-containing stage. This again differs from the results of hypophysectomy where there is a complete absence of normal antrum-containing follicles within 4 days after operation, and indicates that FSH is not completely blocked by massive doses of stilbestrol. Table I, j shows that there is an initial decrease in the number of atretic antrum-containing follicles, followed by a marked increase towards the end of the experimental period. This indicates that the follicle-stimulating function is depressed to some extent. The delay in the appearance of increased atresia argues against a direct effect.

Summary. These results support previous evidence that the ovarian structures which normally secrete estrogen, *i.e.*, antrum-containing follicles and corpora lutea, are affected when the estrogen level is raised, presumably via the anterior hypophysis. However, the suppression of formation of new small follicles is evident as a direct effect of stilbestrol on the ovary.

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17250. Male Mice Tolerate Dosages of Pteroylglutamic Acid* Lethal to Females.

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Female mice are much more susceptible than males to high dosages of pteroylglutamic acid (folic acid). Male mice easily tolerate amounts of this material lethal to every female injected. Females receiving sublethal levels of the compound lose more weight and are slower to recover than comparably treated males.

Harned[1] and associates have investigated the pharmacology of folic acid. They reported this compound to be comparatively innocuous at levels much above the therapeutic range.

When 100 to 400 mg per kg were administered intravenously, acute toxicity was observed in mice, rats, rabbits, and guinea pigs. Death was apparently due to obstruction of renal tubules as a result of the precipitation of folic acid. The mouse and the rat were more resistant than the guinea pig and the rabbit. There is no mention in the paper of a sex difference in the response of the animals to the material injected.

Experimental. More than 400 mice of the dba strain were used in these experiments.

Folic acid was administered both in the form of a saline suspension (0.85%) and dissolved in a solution of sodium bicarbonate (3%). The saline suspension was most frequently used. The various amounts given were diluted so that each animal received 0.1 to 0.2 cc per injection. The dosages given were based on a 25 g mouse unless otherwise stated.

Healthy mature dba male and female mice were subjected to single subdermal injections of 1 to 40 mg (saline suspension) of folic acid to test the effect of massive doses on mortality. The results are summarized in Table I.

Autopsy disclosed the usual precipitation of folic acid in the renal tubules as indicated by the yellowish color of the kidneys. The spleen was reduced in size. Weight averages

* Pteroylglutamic acid for this research was furnished by Lederle Laboratories, Inc.

[1] Harned, B. K., Cunningham, R. W., Smith, H. D., and Clark, M. C., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 289.

TABLE I.
Effect of a Single Injection of Folic Acid on Mortality of Male and Female dba Mice.

Dosage (mg per 25 g mouse)	Male		Female	
	No. injected	No. dead	No. injected	No. dead
1	3	0	3	0
3	3	0	3	0
5	10	1	15	3
10	8	2	11	9
15	7	0	7	7
20	8	0	8	8
40	4	0	4	4

of spleens from female mice receiving 10 mg of folic acid and spleens from control females showed an average reduction of 40% in the spleen weights of the experimental mice. The visceral effects in the male were similar to those observed in the female.

Male and female mice of the same initial body weight were given sublethal injections of folic acid and the effect on the body weight recorded over a period of time.

One group consisted of 20 male and 20 female mice not yet fully mature so that the initial body weights could be exactly matched. Each mouse received a single injection of 5 mg folic acid. The females showed a 10% loss in body weight and had not completely recovered 12 days after the administration of the compound, while the males showed only slight loss of weight followed by rapid recovery and gain in weight.

In other tests mature male and female mice manifested the same sex difference in their response to sublethal injections of folic acid.

In one experiment 6-week-old male and female mice were given single injections of 5 mg folic acid. The males continued to gain weight but more slowly than normal. They gained an average of 3 g in the week following the treatment. The females, one week after the injection weighed an average of 1 g less than their initial weight indicating they were seriously affected.

Discussion. Why the female should be so much more susceptible than the male to high dosages of folic acid has not been determined.

So far as could be discovered no other compound has ever been reported which manifests such a sex difference in its pharmacology.

Summary and conclusion. Male mice are much more resistant than female mice to injections of high dosages of folic acid.

Male mice were only slightly affected by single subdermal injections of 5 mg of folic acid. Female mice receiving the same dosage averaged a 10% loss in body weight followed by a slow recovery.

There were no deaths in a group of male mice receiving 15 to 40 mg in a single injection of folic acid. The same dosages administered to female mice were rapidly lethal in every instance.

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17251. Mechanism of Proteinuria. II. Identity of Urinary Proteins in the Rat Following Parenteral Protein Injection.

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In considering the proteinuria produced by Addis and associates[1] in the rat by intraperitoneal injections of protein, it seemed logical to conjecture that the urinary protein might be identical with the injected protein. Thus, after injections of bovine albumin, it was supposed that the urinary protein, appearing after large doses and some lapse of time, was largely bovine albumin. On the other hand, after injections of egg white and Bence-Jones protein, it was conjectured that the small molecules might more easily and quickly permeate the glomerular membrane, so that protein appeared in the urine rapidly and after smaller dosage.

Doubt was cast upon this desirably simple hypothesis by work recently reported[2] which showed, by the use of hemoglobin as an indicator, that administration of bovine albumin, under the conditions used by Addis, not only saturated the tubular capacity for reabsorbing hemoglobin, but also altered the glomerular permeability to hemoglobin. In contrast,[3] administration of egg white affected hemoglobin excretion in a very different manner. In this study it was possible to demonstrate a similar functional difference between the effects of bovine albumin and egg albumin upon rat serum protein excretion.

Methods. Rabbit antisera to bovine albumin, egg albumin and rat serum were prepared according to the general plan of Goettsch and Kendall.[4] Each female Belgian rabbit received 1.0 mg of antigen intravenously in a volume of 0.1 cc, 3 times a week for 6 weeks. The rabbits were then exsanguinated and the active antisera against a given antigen were pooled and stored in the frozen state. Small portions for current use were stored at 0-2°C.

For the precipitin test, 1 cc of antiserum was added to 2 cc antigen diluted with 0.85% sodium chloride. Results were read after 2 hours at room temperature (25-28°C) followed by refrigeration at 0-2°C for 17 hours. There was no cross precipitation between the antisera and heterologous antigens.

For the quantitative determinations, known amounts of antigen protein measured by the gravimetric method of Barnett, Jones and Cohen,[5] were mixed with antiserum. The precipitates which formed in several tubes with a given antigen and antiserum were pooled and washed repeatedly with 0.85% sodium chloride solution at 0-2°C. Precipitation was performed in the range of marked antibody excess, determined by the addition of antigen to the supernatant fluid and reincubation.

The washed precipitate was drained, then dissolved in a known volume of 4% sodium hydroxide. Ultraviolet light absorption of this solution was determined in a Beckmann spectrophotometer at 280 mμ.†[6] For the latter purpose, serial dilutions were prepared with 4% sodium hydroxide.

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1 Addis, T., Final Report, Contract 338, Office of Scientific Research and Development, Committee on Medical Research, 1946.

2 Lippman, R. W., *Am. J. Physiol.*, 1948, **154**, 532.

3 Lippman, R. W., data to be published.

4 Goettsch, E., and Kendall, F. E., *J. Biol. Chem.*, 1935, **109**, 221.

5 Barnett, C. W., Jones, R. B., and Cohn, R. B., *J. Exp. Med.*, 1932, **55**, 683.

† Use of this procedure was suggested by Dr. Erwin Haas.

6 Hogness, T. R., and Potter, Van R., *Ann. Rev. Biochem.*, 1941, **10**, 509.

TABLE I.
Precipitin Tests Performed upon Rat Urine after Parenteral Injection of Bovine Albumin.

Rat No.	Total protein excretion, mg/24 hr	Urine dilution	Precipitin tests: Bov. alb. antiserum 0 to 4 +	Precipitin tests: Rat serum antiserum 0 to 4 +
62	842	1:24	4	4
35	1440	1:40	1	2
61	859	1:24	2	2
80	320	1:8	2	2
53	750	1:16	2	2
00	106	1:20	3	1
02	202	1:50	2	2
04	404	1:100	3	2
08	270	1:50	4	2
15	1197	1:200	4	1
17	414	1:100	3	2
21	274	1:50	4	1
25	151	1:20	3	2
30	548	1:100	4	1
35	358	1:50	3	2
50	529	1:100	3	2
65	468	1:100	3	2

TABLE II.
Precipitin Tests Performed upon Rat Urine after Parenteral Injection of Egg Albumin.

Rat No.	Total protein excretion, mg/24 hr	Urine dilution	Precipitin tests: Egg alb. antiserum 0 to 4 +	Precipitin tests: Rat serum antiserum 0 to 4 +	Urine dilution	Precip. test rat serum antiserum 0 to 4 +
38a	151	1:20	3	0		
26	158	1:20	2	0		
29	168	1:20	3	0		
31	145	1:20	3	0		
32	139	1:10	3	0		
59	188	1:6	4	0		
25	258	1:250	3	0	1:10	0
38b	88	1:100	2	0	1:10	0
67	234	1:250	4	0	1:10	0
64	195	1:200	3	0	1:10	0
51	80	1:100	4	0	1:10	0
72	192	1:200	3	0	1:10	1
20	208	1:200	4	0	1:10	1
92	139	1:150	3	0	1:10	1
24	90	1:100	3	0	1:10	1
77	18	1:20	3	0	1:10	0
52	184	1:200	4	0	1:10	0
22	225	1:200	3	0	1:10	1

As the light absorption coefficient is specific for each protein, it is necessary to determine the coefficient for each protein or mixture in constant ratio that is used. It was found that the degree of absorption for bovine albumin and egg albumin bore a linear relation to concentration, in accordance with Beer's Law. Highly variable results were obtained with the precipitates formed with rat serum antigen. This was attributed to inhomogeneity of the antigen, and this antiserum was not used for quantitative determinations.

Urine sample were tested qualitatively by precipitation with the appropriate antiserum in the zone of marked antibody excess. For quantitative determinations, precipitation was performed as described, the precipitate was dissolved in 4 cc of 4% sodium hydroxide, after washing and draining, and the light

TABLE III.
Protein Excretion in Rats after Parenteral Bovine Albumin Injections.

Rat No.	Total prot. excretion, mg/24 hr	Total prot. excretion, mg/min.	Bovine alb. excretion, mg/min.	Ratio of bov. alb. to total prot. excreted
35	1440	1.000	0.738	0.74
53	750	0.521	0.369	0.71
61	859	0.597	0.366	0.62
62	842	0.585	0.408	0.70
80	320	0.222	0.167	0.75
45	634	0.441	0.245	0.56
47	918	0.638	0.351	0.55
71	884	0.614	0.398	0.65
74	737	0.512	0.293	0.57
98	1044	0.726	0.508	0.70
00	1160	0.806	0.534	0.66
Mean	872			0.66 ± 0.05

TABLE IV.
Protein Excretion in Rats after Parenteral Egg Albumin Injections.

Rat No.	Total prot. excretion, mg/24 hr	Total prot. excretion, mg/min.	Egg alb. excretion, mg/min.	Ratio of egg alb. to total prot. excreted
26	158	0.1101	0.1000	0.91
29	168	0.1163	0.1257	1.08
31	145	0.1009	0.0923	0.92
32	139	0.0967	0.0822	0.85
38	151	0.1048	0.1090	1.04
59	188	0.1303	0.1081	0.83
95	266	0.1847	0.1712	0.93
96	201	0.1399	0.0782	0.55
07	174	0.1210	0.0939	0.78
08	288	0.2002	0.1880	0.95
10	252	0.1750	0.1782	1.02
14	310	0.2153	0.2295	1.06
Mean	203			0.91 ± 0.10

absorption measured. All determinations were performed in duplicate, and many in quadruplicate on more than one occasion. Total protein concentrations in the urine were determined by the biuret method.[7]

In studying the proteinuria following bovine albumin injections, we followed the same routine described by Addis and used in our previous work. Female rats weighing about 200 g received intraperitoneal injections of 6% bovine albumin in 0.85% sodium chloride solution. The injections, each of 16 cc, were given at 9:30 A.M., 4:30 P.M, and 9:30 A.M. on the next day. At the time of the last injection they were taken from stock diet and given 10% dextrose solution in 0.4% sodium chloride, in order to promote diuresis. Urine was collected from 10:00 A.M. to 3:00 P.M.

Because of differing dose-time relationships, in the experiments with egg albumin it was necesary to use other conditions in order to collect urine at the height of proteinuria. Female rats of similar size were taken from stock diet at 3:30 P.M. and were given a single intraperitoneal injection of 6% egg albumin in 0.85% sodium chloride solution. These rats were then placed on the dextrose-salt solution diet and urine was collected until 9:30 A.M. the next morning. These rats appeared edematous but otherwise well, with large urine volumes.

Results. The results are given in Tables I to IV. It is readily seen that, after injections of bovine albumin, appreciable amounts of rat serum protein are also excreted. On the contrary, after injections of egg albumin, nearly all of the urinary protein is egg albumin, so that qualitatively weak positive reactions to rat serum antiserum are encoun-

[7] Kingsley, G. R., *J. Biol. Chem.*, 1939, **131**, 197.

tered only in very low dilutions, and, quantitatively, the amounts of rat serum protein appearing (calculated by difference) are within the order of magnitude for normal protein excretion.

The data presented here cannot answer whether rat serum protein is excreted along with bovine albumin as the result of diminished tubular reabsorption or the result of increased glomerular permeability or filtration rate. Such information might be obtainable from further investigation under different conditions, varying the rates of protein excretion over a wider range. However, the data given do demonstrate a gross difference in the mechanism of proteinuria following administration of bovine albumin and egg albumin, proteins that differ widely in structure. This difference is consistent with differences found in the effect produced by intraperitoneal injections of these proteins upon hemoglobin excretion in the rat,[2,3] and is of special significance in that the data given here were obtained by an independent experimental approach.

Summary. 1. Parenteral injection of egg albumin in the rat produces proteinuria composed of egg albumin, with no appreciable excretion of rat serum protein.

2. After parenteral injection of bovine albumin, large quantities of rat serum protein are excreted in addition to bovine albumin.

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17252. Some Observations on Growth Factors Required by *Leuconostoc citrovorum*.*

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The presence of a growth factor for *Leuconostoc citrovorum* 8081 in various natural materials was reported by Sauberlich and Baumann.[1] They noted that the organism would respond to thymidine but it was concluded that some other active factor was present in a liver concentrate. The "citrovorum factor" in liver extract was differentiated[2] from a factor active for *Lactobacillus leichmannii* in liver extract by observing that the two factors migrated in opposite directions in an electric field. In the present report, further observations of the characteristics of the "citrovorum factor" are described.

Experimental. Lactobacillus leichmannii 313 (ATCC 7830) and *Leuconostoc citrovorum* 8081 were used in this study. Methods for the use of *Lactobacillus leichmannii* have been previously described;[3-5] the assay technic with *Leuconostoc citrovorum* followed that of Sauberlich and Baumann.[1]

The data of Table I indicate the response of the test organism to concentrated liver extract (15 U.S.P. units per cc), vitamin B_{12} and thymidine before and after heating with alkali. *Lactobacillus leichmannii* gave heavy growth with only 0.03 μl of untreated liver extract, but after treatment with alkali much higher levels of liver extract were required to promote good growth of the organism. As noted elsewhere this alkali treatment destroys the growth-promoting action of vitamin B_{12}

* We are indebted to Dr. J. O. Lampen for thymidine, to Dr. E. E. Snell for hypoxanthine desoxyriboside, and to Dr. E. Hoff-Jorgensen for guanine desoxyriboside.

1 Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, **176**, 165.

2 Lyman, C. M., and Prescott, J. M., *J. Biol. Chem.*, 1949, **178**, 523.

3 Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

4 Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, 1948, **176**, 1465.

5 Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffmann, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

TABLE I.
Effect of Alkali on Growth Promoting Action of Concentrated Liver Extract, 15 U.S.P. Units per cc, for *Lactobacillus leichmannii* and *Leuconostoc citrovorum*.

Additions to 2 ml medium	Optical density*			
	Lactobacillus leichmannii		*Leuconostoc citrovorum*	
	(a)	(b)	(a)	(b)
None	0.28	0.28	0.03	0.03
0.03 μl liver extract	1.15	0.46	0.08	0.08
0.1 " " "	1.33	0.66	0.20	0.19
0.3 " " "	1.32	0.90	0.42	0.42
1.0 " " "	1.39	1.25	0.94	0.92
3.0 " " "	1.40	1.42	1.45	1.50
10.0 " " "	1.50	1.50	1.90	1.90
10 mγ vitamin B_{12}	1.40	0.40	0.03	0.03
10 γ thymidine	1.20	1.00	0.32	0.34

* Determined after 20 hr incubation.
(a) Untreated supplements.
(b) Supplements steamed for 30 min. with 0.2 N NaOH.

for *L. leichmannii,* although thymidine appears to be resistant.[6] The small residual effect of liver extract for *L. leichmannii* after alkali treatment is presumably due to thymidine and other desoxyribosides. In contrast to the results with *L. leichmannii,* the response of *Leuconostoc citrovorum* 8081 to liver extract was unchanged following treatment with alkali and it was also noted that *L. citrovorum* did not respond to vitamin B_{12}. In accord with the results of Sauberlich and Baumann[1] it was found (Table I) that *L. citrovorum* gave only a partial growth response with thymidine as compared to a marked response with liver extract, thus supporting the view that there is an unknown "citrovorum factor", in addition to thymidine, in liver extract. The data of Table I demonstrate that this factor is not identical with the factor required by *Lactobacillus leichmannii,* and show that the "citrovorum factor" is stable to steaming with 0.2 N NaOH for 30 minutes. Lyman and Prescott[7] have noted that the "citrovorum factor" is stable to alkali.

The report that high levels of pteroylglutamic acid (PGA) could in the presence of purine bases replace the "citrovorum factor,"[8] and the finding that 4-amino pteroylglutamic acid, a PGA antagonist, produces an inhibition of the growth of *L. citrovorum* which is reversed by natural materials containing the "citrovorum factor,"[8] prompted further study of the role of PGA in the nutrition of *L. citrovorum.* The data of Table II show that although after 36 hours incubation thymidine or PGA when assayed singly can only partially replace the factor required by this organism, the simultaneous addition of thymidine and PGA to the medium resulted in marked growth after only 18 hours incubation. These results give additional indication of a relationship between PGA, thymidine and the "citrovorum factor." In other experiments, it was found that the addition of 10 γ of vitamin B_{12} per 2 ml medium to tubes containing thymidine and PGA had no additional effect on growth. The addition of *p*-aminobenzoic acid, pteroyltriglutamic acid, pteroic acid or xanthopterin to tubes containing thymidine (Table II) in no instance gave the marked effect on growth produced by the mixture of thymidine and PGA. It was also noted that after a 12-hour incubation period the response of the organism to liver extract was much greater than the response to thymidine *plus* PGA. This finding suggests that the pre-formed factor was present in liver extract, but that in the tubes containing thymidine and PGA some additional transformation must have occurred. However, it is apparent that the presence of thymidine and PGA in natural materials could have a marked

[6] Hoffmann, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C., and Jukes, T. H., *J. Biol. Chem.,* in press.

[7] Lyman, C. L., and Prescott, J. M., *Fed. Proc.,* 1949, **8**, 220.

[8] Sauberlich, H. E., *Fed. Proc.,* 1949, **8**, 247.

TABLE II.
Relationships Between Thymidine, Pteroylglutamic Acid, and Related Compounds for Growth of *Leuconostoc citrovorum*.

Additions to 2 ml medium	Optical density after incubation time of 12 hr	18 hr	36 hr
None	0.04	0.04	0.06
10 γ thymidine	0.08	0.17	0.98
10 γ pteroylglutamic acid (PGA)*	0.04	0.04	0.38
10 γ thymidine + 10 γ PGA	0.40	1.40	1.70
10 γ thymidine + 10 γ p-aminobenzoic acid	0.04	0.15	0.94
10 γ " + 10 γ pteroyltriglutamic acid	0.04	0.20	1.40
10 γ " + 10 γ pteroic acid	0.04	0.15	1.02
10 γ " + 10 γ xanthopterin	0.04	0.15	0.96
10 μl liver extract	1.10	1.80	1.80

* Purified PGA containing at least 98.8% PGA (moisture free basis) was used in these experiments.

TABLE III.
Determination of R_f Values of Fractions of a Liver Extract, 15 Units per cc Separated by Paper Chromatography Using *Lactobacillus leichmannii* and *Leuconostoc citrovorum* as Indicators.

Substance chromatographed	Zones	R_f values of growth factors: *Lactobacillus leichmannii*	*Leuconostoc citrovorum*
Liver extract	1	0.05	—
	2	0.45	—
	3	—	0.55
	4	0.60	0.64
	5	0.72	—
Vitamin B_{12}	1	0.07	—
Thymidine	1	0.63	0.64
Mixture of guanine desoxyriboside and hypoxanthine desoxyriboside	1	0.42	—

effect on the response of *L. citrovorum*.

Liver extract was separated into a number of fractions by paper-strip chromatography following a technic similar to that of Winsten and Eigen.[9] A mixture of 9 parts n-butanol: 1 part acetic acid was used as the mobile phase. The strip chromatograms were laid on a nutrient agar suitable for the growth of the test organisms, the agar was seeded with the appropriate organism, and incubated. The zones of growth indicated the positions of the growth factors present in the liver extract. Chromatograms of known compounds gave R_f values of aid in identifying the fractions separating in liver extract. Table III summarizes the R_f values found on chromatographing liver extract using *Lactobacillus leichmannii* and *Leuconostoc citrovorum* as test organisms. In accord with recent investigations[4,10,11] *L. leichmannii* responded to fractions in liver corresponding to vitamin B_{12} (R_f 0.05), guanine and hypoxanthine desoxyribosides (R_f 0.45), thymidine R_f 0.60), and an as yet unidentified component (R_f 0.72). Of great interest in the present investigation was the finding that *L. citrovorum* not only responded to thymidine (R_f 0.64) but also to another component presumably the "citrovorum factor" (R_f 0.55) in the liver extract. No zones of growth of *L. citrovorum* were observed in the positions on the chromatogram corresponding to vitamin

[9] Winsten, W. A., and Eigen, E., *J. Biol. Chem.*, 1949, **177**, 989.

[10] Kitay, E., Snell, E. E., and McNutt, W. S., *J. Biol. Chem.*, 1949, **177**, 993.

[11] Hoff-Jorgensen, E., *J. Biol. Chem.*, 1949, **178**, 525.

B_{12} and the desoxyribosides of guanine and hypoxanthine. These observations are in full accord with direct growth experiments (Table I).[10] *L. leichmannii* showed no zone of growth on the chromatogram in the position where the "citrovorum factor" was located. Thus the data of Table III provide additional evidence for the separate identities of the factors required by *L. leichmannii* and *L. citrovorum* and demonstrate the existence in liver of an unknown component, not identical with thymidine, that promotes the growth of *L. citrovorum.*

Liver extract, treated with alkali as described in Table I gave results in a paper strip chromatogram identical with those shown in Table III except that vitamin B_{12} was destroyed as indicated by lack of growth of *L. leichmannii* in the region appropriate to vitamin B_{12}.

Summary. 1. *Leuconostoc citrovorum* was found to respond to a growth-promoting factor in the concentrated liver extract, but this organism did not respond to vitamin B_{12}. The alkali-stable nature of the "citrovorum factor" further contrasts it with vitamin B_{12}. 2. Two fractions were separated from liver extract by paper strip chromatography; one of these fractions was presumably thymidine and promoted growth of *Lactobacillus leichmannii* and *Leuconostoc citrovorum.* The other fraction was inactive for *L. leichmannii* but active for *L. citrovorum.*

3. Although thymidine or high levels of pteroylglutamic acid (PGA) when tested singly were only partially effective in promoting growth of *L. citrovorum,* the simultaneous addition of thymidine plus PGA produced marked growth of the organism. This finding suggests a functional relationship between thymidine, PGA and the "citrovorum factor."

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17253. The Lipotropic Effect of Estrogenic Hormones in Inbred Rats.*

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It has been shown[1,2] that estrogenic hormones exert in rats distinct lipotropic activity. In particular, estrogenic compounds (estrone, estradiol benzoate, ethinyl estradiol) allow a more efficient use of methionine as a lipotropic agent. In these studies, various possible sources of error were taken in consideration, such as difference in food intake, weight, sex and genetic identity. Further, it has been found necessary to run control and treated rats simultaneously in all experiments. This latter precautionary measure was prompted by the observation that experimental groups when not run simultaneously often differed in their absolute response even under identical experimental conditions.

With the exception of a few experiments in which unidentified strains obtained from a local dealer were employed, the studies were carried out on rats of the Sprague-Dawley strain.[1,2] In view of the observation that various inbred strains may vary more than 500% in their ability to inactivate estrogen[3] it became necessary to extend the observations on the lipotropic activity of estrogens to other strains beyond those used in previous studies,[1,2] especially to inbred strains, such as the Fischer strain, which shows "both a high threshold for vaginal estrus and an im-

* This work was supported by the Commission on Liver Disease of the Army Epidemiological Board, Preventive Medicine Division, Office of the Surgeon General, Washington, D.C.

1 György, P., Rose, C. S., and Shipley, R. A., *Arch. Biochem.*, 1947, **12**, 125.

2 György, P., Rose, C. S., and Shipley, R. A., *Arch. Biochem.*, 1949, **22**, 108.

3 Segaloff, A., and Dunning, W. F., *Endocrinology*, 1946, **39**, 289.

paired ability for hepatic estrogenic inactivation".[3] In connection with the established lipotropic activity of estrogens in the strains hitherto tested[1,2] the claim that in the Fischer strain "extensive fatty infiltration of the liver develops when massive doses of estrogen are administered" (alpha-estradiol) appeared to be of particular interest. Thus, the possibility that the response of the inbred Fischer strain to estrogens might differ from that previously established in other strains, made it desirable to test the effect of estrogen on rats of this strain kept on an alipotropic diet.

Experimental. The same general procedures were followed as were previously reported.[1,2] Young adult rats, weighing 120-180 g, were used. All of the ahimals were bred and raised in our laboratory from rats of the Fischer strain received, through the courtesy of Dr. A. Segaloff, from Dr. W. F. Dunning (Wayne University, College of Medicine, Detroit). As usual, control and treated animals were run simultaneously in all experiments. When all of the animals of an experiment could not be run at one time, equal numbers of the various groups were started together. All of the animals received the same alipotropic diet which was of low protein-high fat type. It, and the vitamin supplements, were described in the previous paper.[1] In the experiments with ethinyl estradiol the hormone reduced the appetite of the rats to such an extent that it was necessary to run pair-fed controls.

As estrogens, estrone and ethinyl estradiol† were used, given orally,[2] the daily dose being dissolved in 2 drops of cottonseed oil. Control animals were given 2 drops of oil daily. When methionine was given, the daily dose (50 mg dl-methionine),‡ was dissolved in 1 ml of water, and mixed with the vitamin B supplement. In certain of the experiments, where a number of the animals did not drink this mixture, it was given to all by stomach tube.

At the end of the 21 day experimental period, the animals were sacrificed and the liver analyzed for total lipide with the same technique used in the previous experiments.

Results and Discussion. The results obtained are summarized in the attached Table. The most conspicuous difference in the reactivity of this inbred strain in comparison with that of other strains manifested itself in the very high fat content of the liver in the untreated animals fed the basal alipotropic diet. The average fat content of the liver both in male and in female controls of the inbred strain was found to be above 30% while with other strains in previous work[1,2] the corresponding figures were, as a rule, lower than 25%, and frequently less than 20%.

According to our observations made earlier, as well as to reports in the literature,[4] even within one experimental group the fat content of the liver shows often very considerable variations, with a distribution curve of wide extremes. In contrast, animals of the inbred Fischer strain of the present series showed remarkable uniformity of response. For instance, among 18 control animals of Experiments 21 and 22 (Table) only one rat showed a relatively low discrepant figure for liver fat, 14.6%, while the figures for all remaining 17 rats varied only between 30.8 and 43.8%, an exceptionally narrow range for this type of experiment.

Even with the use of the Fischer strain the lipotropic effect of estrogens could clearly be demonstrated, but only with ethinyl estradiol and not with the much weaker estrone, at least not with the doses of estrone given. Addition of methionine increased the lipotropic effect of ethinyl estradiol considerably but did not accentuate the effect of estrone. Even in genetically less homogeneous strains of rats estrone acted as a weak lipotropic agent or was found to be devoid of any lipotropic activity.[1,2] It is probable that in the highly inbred Fischer rats, kept on the basal alipotropic diet, the development of very intensive fat infiltration of the liver may exert a special inhibitory influence as to the manifestation of the lipotropic effect of estrogens. In further consequence, it is not surprising that

† Ethinyl estradiol was kindly supplied by Roche-Organan, Inc., Nutley, N. J.

‡ Kindly furnished by Wyeth, Inc.

[4] Beveridge, J. M. R., Lucas, C. C., and O'Grady, M. K., *J. Biol. Chem.*, 1945, **160**, 505.

TABLE I.
Liver Fat Experiments on Fischer Rats.

Exp. No.	Date	Group	No. of rats	Sex	Treatment (daily)	Liver		Rat weight		Food intake, g/day
						Wt (g)	Total fat (%)	Initial (g)	Change (%)	
21	10-17-47	a	8	M	—	10.6 ± 0.48	37.4 ± 1.5	164 (152-184)	—10.9 ± 1.3	7.7 ± 0.14
	to	b	9	M	Methionine (50 mg)	9.4 ± 0.79	28.3 ± 2.3	166 (150-182)	— 3.5 ± 1.3	7.6 ± 0.21
	11-18-47	c	9	M	Estrone (30 γ)	11.3 ± 0.55	38.6 ± 0.73	167 (153-182)	—11.8 ± 1.4	7.5 ± 0.21
		d	9	M	Methionine (50 mg) + Estrone (30 γ)	8.4 ± 0.39	22.3 ± 2.5	163 (153-180)	— 3.2 ± 1.5	7.1 ± 0.30
22	11-18-47	a	8	F	Methionine (50 mg)	6.9 ± 0.33	20.6 ± 2.4	158 (148-163)	— 3.6 ± 1.5	6.0 ± 0.13
	to	b	8	F	Methionine (50 mg) + Estrone (30 γ)	7.9 ± 0.69	23.5 ± 3.8	158 (147-170)	— 9.1 ± 1.2	5.4 ± 0.17
	11-29-47									
24	6-12-48	a	10	M	—	7.8 ± 0.40	33.1 ± 2.3	164 (135-196)	—21.0 ± 1.7	4.1 ± 0.24
	to	b	10	M	Ethinyl estradiol (30 γ)	8.0 ± 0.33	26.0 ± 1.3	165 (140-182)	—21.9 ± 1.4	4.2 ± 0.21
	6-25-48									
	8- 2-48	c	10	M	Methionine (50 mg)	6.0 ± 0.45	18.7 ± 2.9	149 (134-160)	—14.0 ± 1.3	4.2 ± 0.28
	to	d	10	M	Methionine (50 mg) + Eth. est. (30 γ)	6.2 ± 0.22	5.7 ± 0.11	152 (139-165)	—16.9 ± 1.4	4.6 ± 0.11
	8-27-48									
25	6-25-48	a	11	F	—	7.4 ± 1.3	31.5 ± 2.2	126 (121-152)	—21.2 ± 1.9	3.6 ± 0.34
	to	b	11	F	Eth. est. (30 γ)	7.4 ± 0.50	28.4 ± 1.7	132 (128-150)	—24.7 ± 2.1	3.7 ± 0.38
	9-17-48									
	9-27-48	c	10	F	Methionine (50 mg)	6.1 ± 0.41	17.6 ± 2.2	140 (117-158)	—10.9 ± 2.2	4.5 ± 0.21
	to	d	10	F	Eth. est. (30 γ) + Methionine (50 mg)	5.5 ± 0.22	8.9 ± 1.6	140 (122-160)	—13.5 ± 1.5	4.4 ± 0.18
	1-17-49									

In experiments 24 and 25, the rats were pair fed.

the lipotropic effect of ethinyl estradiol *plus* methionine appeared to be more pronounced in Fischer rats than seen before[1,2] with rats of genetically different and less homogeneous strains. For instance, in one experiment (Table I, Exp. 24), the average fat content of the liver in the control animals was found to be 33.1%, in the group treated with methionine, 18.7%, whereas in rats treated with methionine *plus* ethinyl estradiol, the perfectly normal figure of 5.7% was reached. It should be kept in mind that in these experiments the animals were pair-fed.

In histological examination§ the testes of rats receiving estrogen showed signs of degeneration with consecutive atrophy. In the control groups the testes exhibited essentially normal histological findings. In female rats receiving estrogens constant estrus was observed in contrast to the control rats and to the animals treated with methionine only, which showed essentially normal estrous cycles.

The exceptionally marked response of rats of the Fischer strain to an alipotropic diet is in good accord with the recently published behavior of the same strain with regard to methionine uptake by liver slices.[5] Compared with an inbred Wistar strain, liver slices obtained from Fischer rats showed a very low uptake of methionine. These observations together with the results here presented should reemphasize the importance of genetic variations in stock laboratory animals when used in this type of experiment.

Conclusions. 1. The fat infiltration of the liver in response to an alipotropic diet is more uniform and more intensive in rats of the inbred Fischer strain than that seen in the past in genetically less homogeneous strains. 2. Estrogen, in particular ethinyl estradiol, when given in combination with methionine, exerts a very marked lipotropic effect with a corresponding reduction of the liver fat to normal values.

§ We are indebted to Dr. Harry Goldblatt (Institute for Medical Research. Cedars of Lebanon Hospital, Los Angeles, Calif.) for the histological examination of the testes.

[5] Rutman, R., Dempster, E., and Tarver, H., *J. Biol. Chem.*, 1949, **177**, 491.

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17254. Effects of Pituitary Adrenocorticotropic Hormone (ACTH) in Children with Non-Addisonian Hypoglycemia.

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Severe chronic hypoglycemia of the non-Addisonian type presents a difficult etiologic and therapeutic problem, unless by good fortune it is found on direct examination of the pancreas to be due to a removable adenoma of the islet cells in that organ, a comparatively rare pathological condition. Dietary control of the blood sugar level, which may be quite successful in some cases of mild "functional" or "recurrent" hypoglycemia, is ineffectual in the severe persistent type. Surgical removal of as much as 80 to 85% of the histologically normal pancreas, a therapeutic procedure of last resort, is likely to give temporary relief only, as in two members of the series of patients included in the present investigation. Cautious use of alloxan in one severe case of idiopathic hypoglycemia appeared to be successful.[1] Despite its hepatotoxic action, this agent deserves further trial but only under the strictest supervision.

Repeated attempts on our part to force the gluconeogenetic action of cortin to a degree sufficient to control non-Addisonion hypoglycemia, while midly encouraging in a few instances, have been unsuccessful in the main. However, the isolation of pituitary adreno-

[1] Talbot, N. B., Crawford, J. D., and Bailey, C. C., *Pediatrics*, 1948, **1**, 337.

corticotropin (ACTH) in purified form by Li, Evans and Simpson[2] and by Sayers, White and Long[3] and the demonstration of its diabetogenic effects in normal adult subjects by Browne[4] and Conn, Louis and Wheeler[5] encouraged us to resume our earlier attempts to counteract abnormal hypoglycemic reactions by the administration of a long-acting hormonal agent having a physiological action antagonistic to that of insulin.

The investigation on the metabolic and clinical effects of ACTH reported here was carried out first on 2 severely afflicted children (B.G., age 1 year, and her brother J. G., age 4½ years) and subsequently on 3 additional cases, (brothers, R.R., age 1 year, D.R., age 2½ years, and Dern. R., 4½ years of age) of the less severe "recurrent" or "functional" type. The infant, B.G., had manifested such frequent and severe hypoglycemic reactions, including numerous convulsions, that she had been subjected to partial pancreatectomy when the use of a variety of therapeutic measures, including frequent feedings of either a high-protein, low-carbohydrate, or a high-carbohydrate diet, had failed to alleviate her condition. An estimated 80 to 85% of her histologically normal pancreas had been extirpated. Although this radical procedure had resulted in restoration of the fasting blood sugar to normal values with complete relief from hypoglycemic symptoms for about three weeks, the hypoglycemic state had recurred shortly thereafter in a degree of severity almost as marked as that observed before the operation. The older brother, J.G., who had followed a similar metabolic and clinical course over a period of 3 years was only slightly better off than B.G., despite the surgical removal in two successive operations of even a greater proportion of his apparently normal pancreatic tissue. The other three patients had likewise had severe hypoglycemic symptoms including convulsions when for any reason their food intake was reduced.

Methods. In order to insure quantitative collections of all urine and feces as well as accurate accounting of the amounts of the special dietary formula consumed by each patient, special nurses were assigned to the small metabolic ward where the experimental subjects were kept on specially constructed collection frames. The standard semi-liquid diet, calculated to be nutritionally adequate in every respect, contained 42 g of protein, 45 g of fat and 118 g of carbohydrate in each kg. The daily allowance, adjusted to the caloric needs of the individual subject, was given in three equal meals shortly after 7:00 A.M., 1:00 P.M., and 7:00 P.M.

Fasting blood samples for glucose and eosinophil cell counts were obtained routinely at 7:00 A.M. every day or every second day throughout the entire period of study. The potassium and inorganic phosphorus of the serum were determined at the end of each major period. The 24-hour urine samples were analysed while fresh for total nitrogen, uric acid, creatinine, phosphorus, chloride, sodium and potassium throughout the entire period of study and for 11-oxycorticosteroids and 17-ketosteroids during the last two days of the control and ACTH periods. When successfully collected, stools for each major period were analysed for N, P, Cl, Na and K. Standard analytical methods were employed for the various substances named. The "true" blood glucose was determined by Nelson's[6] modification of the Shaffer-Somogyi[7] method, according to which values below 50 mg/100 ml are regarded as hypoglycemic.

In each experiment ACTH* was administered intramuscularly in equal doses (either 9 or 10 mg equivalent to Armour standard preparation LA-1-A) every six hours over a period of 4 days. This test period was preceded by a control- or pre-period of several days length and was followed by a post-

[2] Li, C. H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, 1943, **149**, 413.

[3] Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, 1943, **149**, 425.

[4] Browne, J. S. L., Josiah Macy Jr. Foundation Report, New York, June, 1943.

[5] Conn, J. W., Louis, L. H., and Wheeler, C. E., *J. Lab. Clin. Med.*, 1948, **33**, 651.

[6] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

[7] Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

* Supplied by the Armour Laboratories through the courtesy of Dr. John R. Mote, Medical Director.

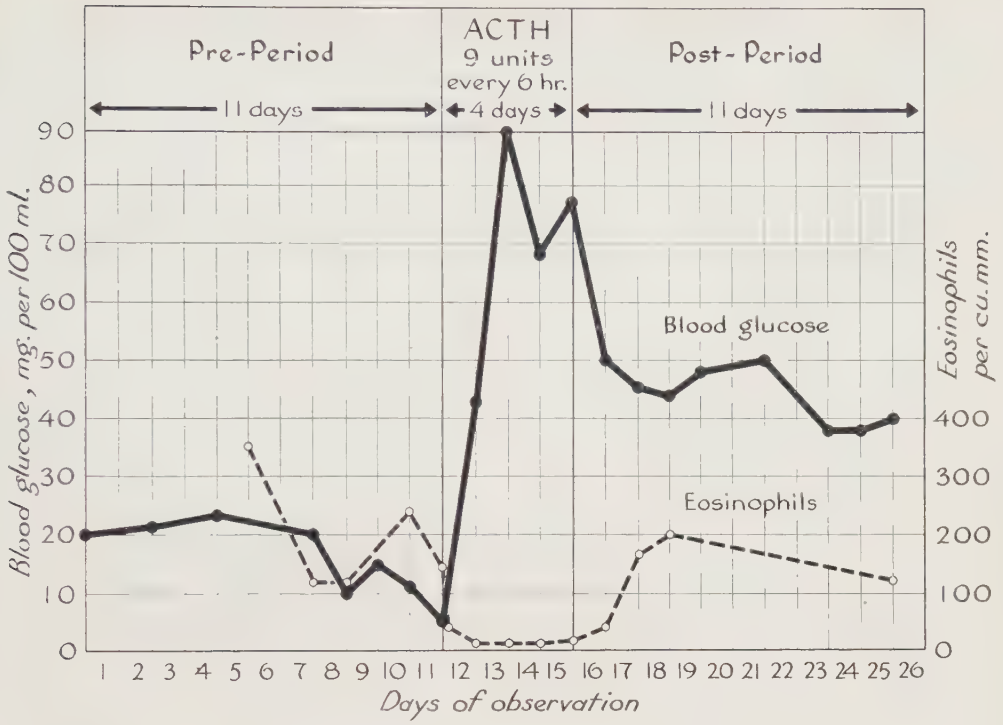

FIG. 1.

Effects of pituitary adrenocorticotrophic hormone (ACTH) on fasting blood glucose and eosinophil count in B.G.

period of similar length. Sugar tolerance tests were performed in the pre-period and again during the latter part of the ACTH period in all cases. In this test blood glucose was determined immediately before and fifteen minutes, 1 hour, 2 hours, 3 hours, 4 hours and 5 hours after termination of the intravenous infusion of a 25% solution of glucose (0.5 g glucose per kg of body wt.). The insulin sensitivity test was carried out by the "insulin-with-glucose" tolerance test proposed by Engel and Scott[8] on the last three patients studied. The ability of the pituitary gland to elaborate or to release ACTH was evaluated in these 3 patients by means of the test proposed by Recant, Forsham and Thorn,[9] according to which a marked decrease in the eosinophil count 4 hours following the intravenous infusion of epinephrine indicates a normal response. The dosage of epinephrine was adjusted to the size of the child. R.R. was given 0.3 mg in 50 ml of saline solution; D.R. 0.4 mg in 60 ml, and Dn. R. 0.5 mg in 70 ml, the infusion running one hour.

Results. The experimental data on the 5 subjects with non-Addisonian hypoglycemia included in this study indicate that their responses to ACTH administration are similar in type to those reported for normal adult subjects.[5,10] Severe hypoglycemia and attendant symptoms were completely abolished during the period of intensive hormone administration and for at least a week after its withdrawal. The sharp fall and the abnormally prolonged low level of blood sugar in the glucose-tolerance curve, which represented the characteristic response of each of the patients during the control period, were

[8] Engel, F. E., and Scott, J. L., *Proc. Amer. Soc. Clin. Invest.*, Atlantic City, N. J., May 2, 1949, p. 17.

[9] Recant, L., Forsham, P. H., and Thom, G. W., *J. Clin. Endocrin.*, 1948, **8**, 589.

[10] Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrin.*, 1948, **8**, 15.

found to be absent during the period of ACTH administration, the curve becoming essentially normal. None of the 5 patients showed sugar in the urine at any time during the entire period of study, except for the mild glycosuria which occurred at the height of the glucose tolerance test made in the ACTH period. Insulin hypersensitivity was likewise counteracted to a large extent by the ACTH in the cases tested. The eosinophil cell counts 4 hours after infusion of epinephrine averaged 31/cmm compared to the average initial count of 133. This 79% fall was interpreted as indicating normal capacity on the part of the pituitary to produce ACTH in the 3 cases tested.

The eosinophil cells of the blood fell precipitately from the normal range (between 100 and 350/cmm) to between 0 and 10/cmm within 4 hours after ACTH was first administered and remained at this low level so long as the hormone was given at 6-hour intervals. While the fasting blood sugar during the post-period tended to remain for a number of days at levels intermediate between the hypoglycemic values of the pre-period and the normal values of the ACTH period, the eosinophils returned to their original normal counts fairly promptly, usually within a day. Responses of the blood glucose and eosinophil cell count to ACTH administration are illustrated in Fig. 1 which presents data obtained on B.G., the most severe case in the series. Changes in the other four cases were very similar to these. The only untoward effects of the ACTH were a transient vasopressor reaction after each injection and a moderate tendency to oliguria during the first day or two of intensive administration. These effects appear to be due to contamination with posterior pituitary hormones.

During a follow-up period of 94 days subsequent to the "post-period" shown in Fig. 1, ACTH was administered to B.G. in a dose of 18 mg once every 2 days. The fasting blood sugar was then determined at the end of each 48-hour period, when it would presumably be at its lowest level. The values so obtained were found to range between 40 and 68 mg/100 ml throughout the period, except for a few days when the patient refused part of her diet because of an upper respiratory infection. Two of the morning values at that time were 24 and 16. Her diet, which was unrestricted, was taken avidly throughout the remainder of the period and the gain in weight was satisfactory. She learned to walk alone in the interval. No clinical signs of hypoglycemia or of toxic side effects were observed at any time. On the contrary, the ACTH appeared to be almost as specific for the control of this severe hypoglycemic disorder as insulin is for the control of diabetes mellitus.

The urinary excretion of 11-oxycorticosteroids and 17-ketosteroids was increased as a result of the ACTH injections by percentages ranging between 75 and 400. At the same time, the uric acid excretion increased 50 to 100%. Whereas intensive administration of ACTH has been reported to induce a negative nitrogen balance in most normal adult subjects, a positive balance was maintained in all periods in these very young, growing subjects, as long as the full diet was taken. However, the magnitude of the positive balance was less during the period of intensive ACTH administration than during the pre- and post-periods. In the one patient, B.G., whose stool, as well as urine, analyses have been completed up to date, the phosphorus balance was likewise slightly positive in all periods. The size of the positive balance during the ACTH period, however, was less than that for the fore-period and the after-period. Sodium and chloride showed no significant tendency to increased retention during the ACTH period, but showed a fairly marked increase in excretion in the post-period. The comparatively low NaCl content of the diet may account in part for the low degree of retention. Potassium showed a small negative balance during the ACTH period but positive balances for the pre-period and the post-period.

There were small but consistent changes in the potassium and inorganic phosphorus of the blood serum of all 5 of the subjects as a result of ACTH administration. In every instance both elements fell to lower levels. The fasting K values, which were slightly elevated in 3 and near the upper limit of normal in 2 of

the cases, were decreased from an average of 5.80 meq/1 in the pre-period to 5.06 meq/1 at the end of the ACTH period. At the same time the P was decreased from an average for the group of 3.03 to 2.79 meq/1. These decreases in serum K and P are interpreted as an indication of glycogen deposition during the ACTH period rather than being due to increased excretion. The magnitude of the decrease in retention of these two elements appeared to be far too small to influence blood composition.

The mucoprotein and the non-specific hyaluronidase inhibitor of the serums of the patients were both found by our colleagues, R.A. Good, V. C. Kelley, and D. Glick, to be increased at the end of the ACTH period by nearly 100% of the control levels. These elevations were transient, however, essentially normal values being found 4 days after withdrawal of the hormone.

Summary. The effects of ACTH on the fasting blood sugar level and glucose tolerance test; on the potassium and inorganic phosphorus content of the serum; on the nitrogen, phosphorus, chloride, sodium and potassium balances; on the urinary excretion of uric acid, creatinine and adrenal corticosteroids and on the blood eosinophil counts were determined in 5 young children with non-Addisonian (familial) hypoglycemia. The type of response to ACTH was similar in all respects to that of the normal adult. However, under the conditions of this experiment, instead of producing a transient state of diabetes mellitus, as it does in the normal subject, the ACTH appeared merely to reverse the hypoglycemic tendency, with return of the fasting blood sugar level and the glucose tolerance curve to normal. While the eosinophil count returned to normal promptly upon withdrawal of ACTH, the blood sugar remained above the threshold for hypoglycemic reactions for at least 10 days without ACTH in the most severe case in the series (Fig. 1). Administration of 18 mg of ACTH in one dose every 48 hours thereafter served to maintain this one-year-old patient in an essentially non-hypoglycemic state for more than three additional weeks. Results of the study suggest that ACTH may prove to be as effective in the control of this non-Addisonian hypoglycemic disorder as insulin is in the control of diabetes mellitus.

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17255. Prevention of Chemotherapeutic Effects of 4-Amino-N^{10}-Methyl-Pteroylglutamic Acid on Mouse Leukemia by Pteroylglutamic Acid.*

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Certain derivatives of pteroylglutamic acid (PGA) which have in common the substitution of an amino group in the 4 position of the pteridine ring have a demonstrable effect against some strains of transplanted mouse leukemia[1–3] and against solid tumors in mice

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† Fellow of The American Cancer Society, recommended by the Committee on Growth of The National Research Council.

1 Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer*, 1949, **2**, 113.

2 Law, L. W., *J. Nat. Can. Inst.*, in press.

3 Burchenal, J. H., Johnston, S. F., Burchenal, J. R., Kushida, M. N., Robinson, E., and Stock, C. Chester, PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 381.

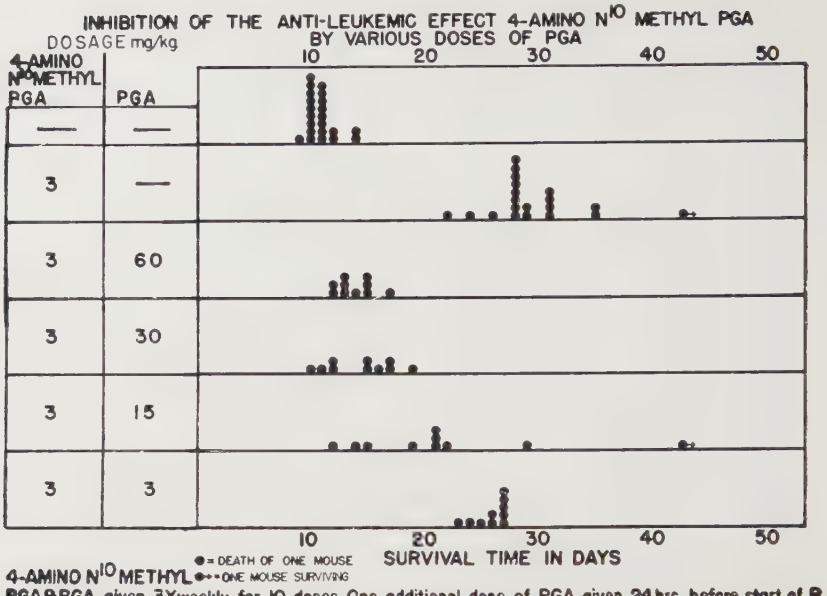

FIG. 1.

and rats.[4-6] The beneficial effects of such compounds in some cases of acute leukemia in children were first reported by Farber *et al.*[7] and have since been confirmed by numerous clinical trials.

In an attempt to elucidate the mode of action of the 4-amino analogs of PGA against leukemic cells, an investigation was undertaken to determine whether prior administration of PGA could prevent the anti-leukemic effects of 4-amino-N^{10}-methyl-PGA.

Experimental. The procedure for inoculating mice of the AKm stock with leukemia Ak 4 was similar to that reported previously.[1,3] 0.1 ml of a saline suspension of leukemic spleen containing 1,000,000 cells was injected intraperitoneally. Forty-eight hours later, treatment with 4-amino-N^{10}-methyl-PGA.‡[8] was started in the maximum tolerated dose of 3 mg/kg 3 times weekly for 10 doses by the intraperitoneal route. Intraperitoneal injections of PGA were started 24 hours after the inoculation of the leukemia and then given one hour before each dose of the antimetabolite. Various dosage levels of PGA were studied against the standard dose of 4-amino-N^{10}-methyl-PGA. As can be seen from Fig. 1 and Table I, PGA administered at levels of 60 and 30 mg/kg by this schedule blocked the anti-leukemic effects of the antagonist. The mice so treated died at approximately the same time as the controls whereas the mice treated with 4-amino-N^{10}-methyl-PGA alone survived at least twice as long as the controls. When the dosage level of PGA was only 15 mg/kg, less blocking of effect was seen and with 3 mg/kg there was almost none.

A less toxic conjugate, pteroyltriglutamic

[4] Schoenbach, E. B., Goldin, A., Goldberg, B., and Ortega, L. G., *Cancer*, 1949, **2**, 57.

[5] Sugiura, K., Moore, A. D., and Stock, C. Chester, *Cancer*, 1949, **2**, 491.

[6] Moore, A. D., Stock, C. Chester, Sugiura, K., and Rhoads, C. P., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 396.

[7] Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., and Wolff, J. A., *New Eng. J. Med.*, 1949, **238**, 787.

‡ We are indebted to Dr. Williams and the late Dr. Subarrow of the Lederle Laboratories for our supply of this compound.

[8] Smith, J. M., Jr., Cosulich, D. B., Hultquist, M. D., and Seeger, D. R., *Trans. N. Y. Acad. of Sc.*, 1948, **82**, 10.

TABLE I.
Prevention of the Anti-leukemic Effect of 4-Amino-N^{10}-Methyl-PGA by Pteroylglutamic Acid.

Experiment	Dose, mg/kg SK 1275	Dose, mg/kg PGA	No. of mice	Survival time in days Range	Mean	S.D.
1	—	—	20	10-18	12	±1.87
	3	60	10	13-24	16	±3.32
	3	—	7	22-41	30	±5.84
2	—	—	18	10-18	13	±1.79
	3	30	20	12-29	17	±1.27
	3	—	19	27-49*	31	±5.3
3	—	—	18	11-17	13	±1.52
	3	60 × 4 30 × 4	17	9-18	16	±2.19
	3	—	11	21-59	30	±9.8
4	—	—	22	9-14	11	±1.22
	3	60	10	12-17	14	±1.51
	3	30	10	10-19	14	±2.86
	3	15	10	12-43†	22	±8.48
	3	3	10	23-28	26	±1.84
	3	—	20	22-43†	30	±4.17

* 2 mice surviving at 49 days.
† 1 mouse surviving at 43 days.

TABLE II.
Prevention of Anti-leukemic Effect of 4-Amino-N^{10}-Methyl-PGA by Pteroyltriglutamic Acid.

Dose, mg/kg 4-Amino-N^{10}-methyl-PGA	Dose, mg/kg PTGA	No. of mice	Survival time in days Range	Mean	S.D.
—	—	20	8-13	10	±1.53
—	400	10	9-12	11	±1.38
3	400	9	12-19	16	±2.64
3	200	10	15-20	18	±1.69
3	100	9	13-24	19	±2.82
3	50	10	13-34*	20	±5.49
3	25	10	17-30	25	±3.89
3	12.5	10	19-30	26	±3.29
3	6.25	9	27-34*	29	±2.23
3	—	19	19-34†	28	±3.79

* 1 mouse surviving at 34 days.
† 2 mice surviving at 34 days.

acid (PTGA)[9] was also tested by this technic for its ability to prevent the anti-leukemic effect of 4-amino-N^{10}-methyl-PGA. Table II shows the effect of this conjugate of PGA. Some prevention of the chemotherapeutic action of a standard course of the antagonist was noted at 50 to 400 mg/kg, but none at 6.25 to 25 mg/kg.

Discussion. 4-amino-N^{10}-methyl-PGA[8] was used in preference to 4-amino-PGA in these experiments since its chemotherapeutic effect against leukemia Ak 4 is greater and more consistent.[1,3] The inhibitory action of this anti-metabolite against *Streptococcus fecalis R* has been shown by Franklin *et al.*[10] to be of high degree, but reversible by approximately equal amounts of PGA over a range of 10 to 10,000 γ per 10 ml of medium. In the weanling Wistar rat on a purified diet supplemented with 10.0 mg/kg of PGA, good growth occurred at 1.0 mg/kg of 4-amino-N^{10}-methyl-PGA, but animals fed 3.0 mg of

[9] Boothe, J. H., Mowat, J. H., Hutchings, B. L., Angier, R. B., Waller, C. W., Stokstad, E. L. R., Semb, J., Gassola, A. L., and Subbarow, Y., *Trans. N. Y. Acad. Sc.*, 1948, **10**, 70.

[10] Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.*, 1949, **177**, 621.

the anti-metabolite per kilo of diet all died. When the dosage of PGA was increased to 100 mg/kg the rats at the 3.0 mg/kg level survived, but no protection was noted at the 10 mg/kg level of anti-metabolite. In the chick, the same investigators[10] found a slight inhibition of growth by 3 mg of the antagonist per kilo of purified diet in the presence of 0.1 mg/kg of PGA. When the level of PGA was raised to 10 mg/kg, this inhibition of growth was prevented. Hertz and Tullner[11] found that with this compound inhibition of the estrogen induced response of the chick oviduct could be obtained by 3 daily subcutaneous injections of 0.2 mg each. Blocking of this inhibition was demonstrated with 3 daily doses of 5 mg each of PGA given subcutaneously one hour prior to the injection of the anti-metabolite.

With a closely related antagonist, 4-amino-PGA, the prevention by PGA of toxicity,[12,13] of inhibition of estrogen induced growth of uterus or oviduct,[11] and of the inhibitory effect against mouse leukemia[2] and solid tumors[4] has been observed.

It has been shown above that prevention of the toxic effects of these 2 analogs of PGA may be obtained within narrow ranges at the minimum toxic dose by high dosage of PGA. Since the dose of 4-amino-N^{10}-methyl-PGA used in these experiments was just below the toxic dose, it was to be expected that an effect obtained at this level might be counteracted by PGA. The ratio of PGA to anti-metabolite necessary to prevent this anti-leukemic effect was approximately similar to that required in other studies.[10,11] The ability of PGA and PTGA to prevent the anti-leukemic effects of this anti-metabolite would seem to lend support to the theory that the chemotherapeutic effect of this derivative is due to its action as a metabolic antagonist of PGA.

Summary. The effect of 4-amino-N^{10}-methyl-pteroylglutamic acid in prolonging the survival time of mice with transplanted leukemia Ak 4 can be blocked almost completely by prior administration of 10 to 20 times as much pteroylglutamic acid and, to a lesser degree, by 17 to 125 times as much pteroyltriglutamic acid.

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11 Hertz, R., and Tullner, W. W., *Endocrinology*, 1949. **44**, 278.

12 Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 398.

13 Philips, F. S., and Thiersch, J. B., *J. Pharm. and Exp. Therap.*, 1949, **95**, 303.

17256. Effect of Aluminum Hydroxide Gel and Calcium Lactate on Serum Bicarbonate.*

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The observations herein reported indicate that subjects with reduced kidney function respond to the oral administration of suitable amounts of calcium lactate and aluminum hydroxide gel with a marked and sustained elevation of serum bicarbonate. This response occurred both in very young infants with physiologically immature kidneys[1] and in older infants and children with kidney disease. No such increase in serum bicarbonate accompanied the administration of comparable amounts of the two agents to older infants and children with normal kidney function.

* This investigation was supported in part by research grants from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and from the New York Heart Association, Inc.

1 Barnett, H. L., Hare, K., McNamara, H., and Hare, R., *J. Clin. Invest.*, 1948, **27**, 691.

TABLE I.
Effect of Aluminum Hydroxide Gel and Calcium Lactate on Serum Bicarbonate.

Subject	Age, days	Wt., kg	Daily oral intake		Serum bicarbonate content,[6] mM/L					
			5% aluminum hydroxide gel, ml/kg	Ca lactate g/kg	Before treatment	During treatment,* days 1	2	3	4	5
				Young infants.						
CA	1	4.2	10	1.6	27.0	26.8	33.5			
OR	2	3.2	7.5	1.5	24.7	26.2	32.3	34.1		
AD	2	3.6	11	1.3	20.9	25.0	29.1		27.3	26.7
FR	7	4.2	7	1.1	19.6		25.8	24.3	29.1	
JO	9	3.3	11	1.7	22.7	25.2	29.1	30.8		
RA	21	2.7	15	1.6	26.3	31.4	30.9	33.8		
NE	28	3.0	7	1.4	25.8	31.9	36.7			
				Children with kidney disease.						
	Years									
MA	1	7.5	10.0	1.5	16.2	20.2	24.0		25.7	26.0
		7.3	10.3	1.0	19.6	25.4	26.7		29.1	32.3
		7.5	10.0	1.0	19.5		24.3	25.5		
GR	14	33.0	3.9	0.6	21.5	25.4	28.3			26.2
				Infants and children with normal kidney function.						
AN	16 mos.	10.2	8.5	1.3	25.7	25.9	25.4	26.5		
UR	23 mos.	6.2	9.0	1.4	25.3	27.3	27.3	25.2		
BO	5½ yrs.	16.0	8.8	1.2	26.8	30.6	27.2	29.2		
AR	8½ yrs.	16.0	9.4	1.3	30.6	30.4		31.5		

* The last value coincides with the final day of treatment with both drugs. Serum bicarbonate values returned to pre-treatment levels within 24 to 48 hours following discontinuation of either or both drugs.

[6] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

The quantities of calcium lactate and aluminum hydroxide gel given and the magnitude and rate of change in serum bicarbonate are shown in Table I.

Given separately, neither agent produced an increase in serum bicarbonate in subjects with either normal or reduced kidney function. Failure of aluminum hydroxide gel to raise serum bicarbonate in subjects with normal kidney function is in accord with earlier reports.[2] Its effect in subjects with reduced kidney function is less well established. Freeman and Freeman[3] gave this drug to children with chronic nephritis but did not report detailed data on serum bicarbonate. They did mention relief from acidosis a few days after adding aluminum hydroxide gel to the low phosphorus diet of a 10 year old boy with chronic renal insufficiency.[4] From our findings, correction of renal acidosis with aluminum hydroxide gel alone would not be expected. The apparent discrepancy may, perhaps, be explained by differences in phosphorus intake since our subjects received milk, and therefore relatively large amounts of phosphorus.

The mechanisms underlying the difference in response of serum bicarbonate in subjects with reduced and normal kidney function to calcium lactate and aluminum hydroxide gel are not yet explained. Detailed balance studies are now in progress in an attempt to define these mechanisms.

The observed increase in serum bicarbonate in subjects with reduced kidney function following oral calcium lactate and aluminum hydroxide gel is of interest in several respects. It is known that aluminum hydroxide gel alone lowers serum phosphate and raises serum calcium in patients with chronic renal acidosis.[3] Our results indicate that calcium lactate given with the aluminum hydroxide gel may correct the acidosis. The possible application of the combined use of the two drugs in the treatment of tetany of the newborn is under investigation. Finally, the fact that elevation of serum bicarbonate occurred in very young infants suggests that the ability of the immature kidney of young infants to excrete excess base may be less well developed than its ability to conserve base.[5] Observations are planned to study this function in young infants directly.

Conclusions. The combined oral administration of calcium lactate and aluminum hydroxide gel produced a marked and sustained elevation of serum bicarbonate in young infants with physiologically low kidney function and in older subjects with impaired function. This effect did not occur in subjects with normal kidney function. Given separately, neither drug produced an increase in serum bicarbonate in subjects with normal or reduced kidney function.

We are indebted to Flora Hurwitz, R.N., for nursing and technical assistance.

[2] Kirsner, J. B., *Am. J. Digest. Dis.*, 1941, **8**, 160.

[3] Freeman, S., and Freeman, W. M. C., *Am. J. Dis. Child.*, 1941, **61**, 981.

[4] Freeman, S., and Freeman, W. M. C., *Quart. Bull. Northwestern U. Med. School*, 1943-45, **17-19**, 275.

[5] Gordon, H. H., McNamara, H., and Benjamin, H. R., *Pediatrics*, 1948, **2**, 291.

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17257. Effect of Febrile Plasma, Typhoid Vaccine and Nitrogen Mustard on Renal Manifestations of Human Glomerulonephritis.

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Remissions have been observed to occur spontaneously in patients with chronic diffuse glomerulonephritis in the nephrotic phase. Blumberg and Cassady[1] and Janeway[2] have reported that patients with diffuse glomerulonephritis after accidental or induced measles show transient reduction in protein excretion and significant diuresis, confirming the generally accepted opinion that remissions in this disease may be occasioned by intercurrent infection. Since the febrile phase of infection may be accompanied by profound disturbance in renal hemodynamics[3,4] it seemed possible that both decrease in proteinuria and diuresis might be directly attributable to alteration in renal hemodynamics.

On the other hand, Becker[5] has shown that administration of HN_2 prevents the development of the Schwartzman phenomenon in rabbits.† Since there is reason to believe that diffuse glomerulonephritis may be the result of immunological alteration in renal tissue, it seemed conceivable that a common factor might be operative in infection and following the administration of these agents. In this view it was conceivable that reactive factors might be present in the plasma of patients with acute infections which would produce remission of the nephrotic syndrome.

To test these possibilities, we have explored the effects of 1) pyrogenic reaction, 2) infusion of plasma from patients acutely ill with pneumococcal and hemolytic streptococcal infections, and 3) one of the nitrogen mustards, methyl bis(B-chloroethyl)amine hydrochloride (HN_2), on protein excretion, diuresis, and rate of glomerular filtration in patients with diffuse glomerulonephritis.

Methods. Observations were made on daily weight, 24 hour urinary volume and urinary protein excretion, rate of glomerular filtration (C_{IN}) as measured by the inulin clearance, and renal plasma flow (C_{PAH}) as measured by the p-aminohippurate clearance in 4 patients with chronic diffuse glomerulonephritis in the nephrotic phase.

Urinary protein was determined by the micro-Kjeldahl and biuret methods[8] and inulin and p-aminohippurate by methods previously described.[9]

Results. The pyrogenic reaction was induced in one patient with marked renal functional impairment on 2 occasions with intravenously administered typhoid vaccine. On both occasions there was marked decrease in proteinuria, accompanied in the one instance in which it was measured by decreased filtra-

* Assistant in Medicine and Fellow in Physiology. Aided in part by grants from the Commonwealth Fund and the New York Heart Association.

1 Blumberg, R. W., and Cassady, H. A., *Am. J. Dis. Child.*, 1947, **73**, 151.

2 Janeway, C. A., Moll, G. H., Armstrong, S. H., Jr., Wallace, W. M., Hallman, N., and Barness, L. A., *Tr. A. Am. Physicians*, 1948, **61**, 108.

3 Chasis, H., Ranges, H. A., Goldring, W., and Smith, H. W., *J. Clin. Invest.*, 1938, **17**, 683.

4 Bradley, S. E., Chasis, H., Goldring, W., and Smith, H. W., *J. Clin. Invest.*, 1945, **24**, 749.

5 Becker, R. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 247.

† Since our investigations were initiated, Janeway *et al.*[6] have reported that experimental nephritis produced in rabbits by the administration of bovine gamma globulin can be prevented by HN_2 and Dammin and Bukantz[7] have shown that HN_2 inhibits antibody production, Arthus reaction, and vascular lesions in rabbits produced by horse serum antigen.

6 Janeway, C. A., Schwab, L., Moll, F. C., Hall, T., and Hawn, C. V., personal communication.

7 Dammin, G. J., and Bukantz, S. C., *J.A.M.A.*, 1949, **139**, 358.

8 Hiller, A., Greif, R. L., and Beckman, W. W., *J. Biol. Chem.*, 1948, **176**, 1421.

9 Goldring, W., and Chasis, H., Hypertension and Hypertensive Disease, Commonwealth Fund, New York, 1944.

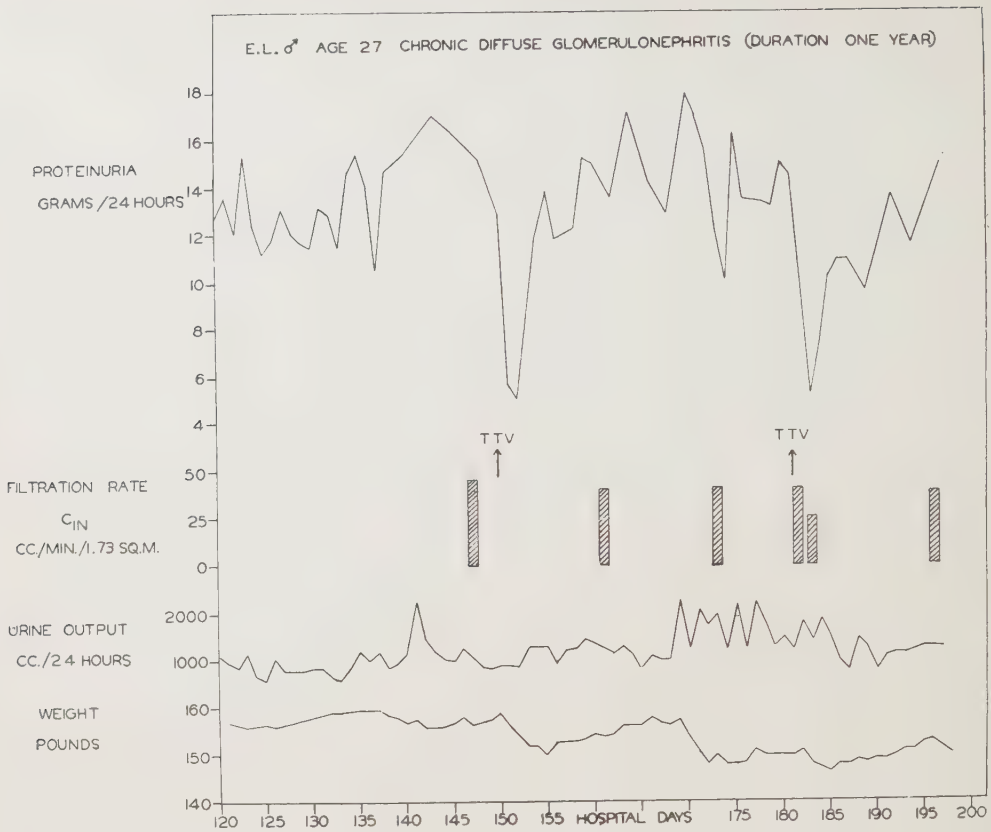

FIG. 1.
Effect of pyrogenic reaction on proteinuria and rate of glomerular filtration.

tion rate. No diuresis occurred (Fig. 1). It is our tentative opinion that the decrease in proteinuria was related to the renal hemodynamic alteration as manifested in part by decrease in filtration rate.

Two patients were given 1975 and 1280 cc of plasma intravenously respectively from patients with hemolytic streptococcal and pneumococcal infections in amounts of 125 to 500 cc at intervals in one patient over a period of one month and in the other over a period of 4 months. In neither patient was there a decrease in proteinuria or diuresis.

HN_2 was administered intravenously to 3 patients in 2 doses of 0.2 mg per kilo on successive days. In all 3 proteinuria decreased. In patient B.D. whose control C_{IN} and C_{PAH} were 13.9 cc and 66.3 cc per minute respectively, daily excretion of protein during a control period of 28 days ranged from 21.4 to 14.5 g, averaging 17.0 g. Protein excretion decreased to 2.3 and 10.6 g in the 24 to 48 and the 48 to 72 hour periods following the administration of HN_2, after which it returned to the pretreatment level. Diuresis did not occur. In patient L. G. whose control C_{IN} and C_{PAH} were 10.9 and 236 cc per minute, the daily excretion of protein during a control period of 113 days ranged from 9.1 to 4.2 g, averaging 7.0 g. Following administration of HN_2 proteinuria decreased to 4.0 g per day, the decrease being first observed during the first 24 hours of HN_2 administration, and persisting for 4 days. Diuresis did not occur.

Patient J.H., whose control C_{IN} and C_{PAH} were 116 and 839 cc per minute, was given HN_2 on 2 separate occasions. As shown in Fig. 2, in each instance there occurred diuresis and marked decrease in protein-

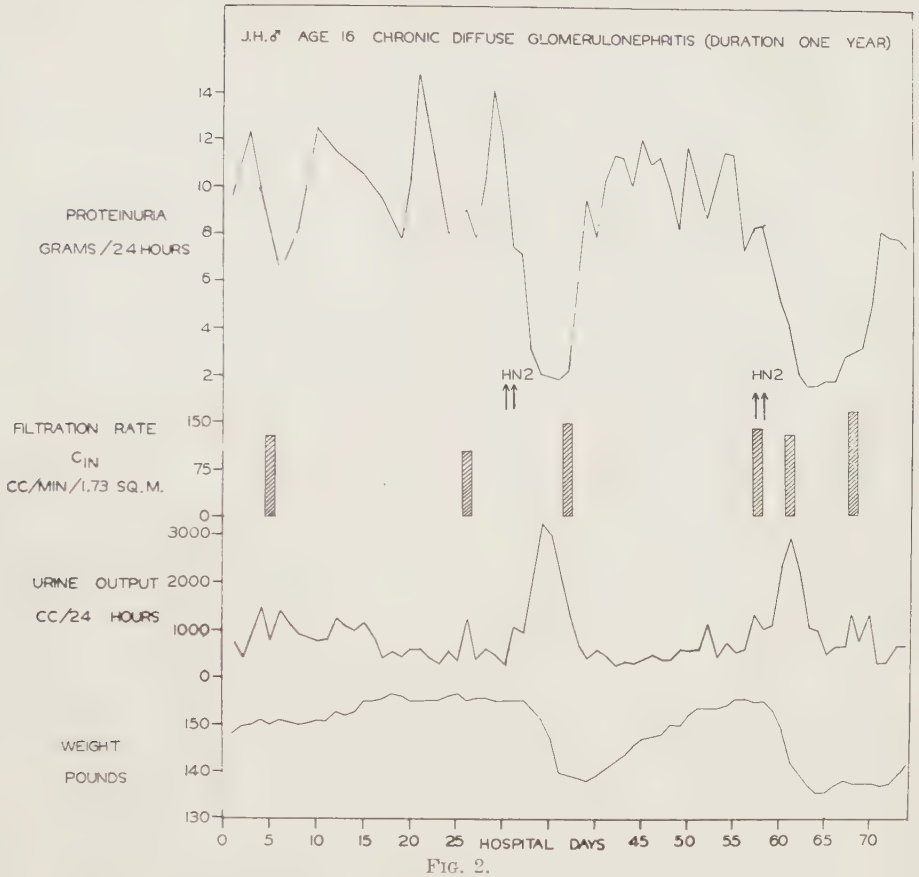

FIG. 2.
Effect of nitrogen mustard on proteinuria and rate of glomerular filtration.

uria, accompanied by an increased filtration rate. The diuresis was associated with disappearance of edema.

Summary. Administration of plasma from patients acutely ill with pneumococcal and streptococcal infections failed to decrease proteinuria or induce diuresis in 2 patients with chronic diffuse glomerulonephritis.

Induction of the pyrogenic reaction was accompanied by decrease in proteinuria on 2 occasions in one patient. We are inclined to attribute this result to the concomitant decrease in the rate of glomerular filtration.

Therapeutic doses of HN_2 reduced proteinuria but diuresis failed to occur in 2 patients with advanced chronic diffuse glomerulonephritis with marked renal functional impairment. However, in one patient with minimal renal functional impairment, administration of HN_2 was followed on 2 separate occasions by diuresis, marked reduction in proteinuria and concomitant increase in filtration rate, a combination of effects consistent with a return of glomerular function towards normal.‡

Our observations indicate that reversal of renal manifestations of human glomerulonephritis can be induced by HN_2.

This study is being extended to include patients in earlier phases of glomerulonephritis.

‡ It is of interest in this connection that in dogs the filtration rate may be substantially increased 40 to 72 hours after intravenous administration of HN_3.[10]

[10] Houck, C. R., Crawford, B., Bannon, J. H., and Smith, H. W., *J. Pharm. and Exp. Therap.*, 1947, **90**, 277.

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17258. Occurrence of a Transient Leucocytosis During the Jarisch-Herxheimer Reaction.*

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There is no agreement in the medical literature on the characteristic blood picture in untreated early syphilis. Willcox[1] in a study of 405 patients with early syphilis found an average total white blood count of 8,950 with a normal differential. Previous to this report, findings of leucocytosis,[2] lymphocytosis,[3] monocytosis,[4] and eosinophilia[5] fill the literature on this subject.

No report exists, as far as can be determined, concerning the changes in the peripheral blood picture during a Jarisch-Herxheimer reaction. Sheldon and Heyman[6,7] have reported the occurrence of an acute polymorphonuclear leucocyte infiltration around early syphilitic lesions during a Jarisch-Herxheimer reaction, but they make no mention of the peripheral blood picture.

Material. Seventeen patients with early syphilis were used in this study. Of these 3 were seronegative primary, 6 were seropositive primary, and 8 were secondary. Four patients were female and 13 were male.

Method. After a diagnosis of syphilis was made and the pre-treatment white blood counts, differentials, and base line temperatures were completed, the patients were given a 300,000 unit dose of aqueous penicillin-G intramuscularly. They were then followed at 2 hourly intervals with total white blood counts, differentials and rectal temperatures. A total of 8 patients were followed with differential counts throughout the entire course of the reaction. All white counts were done by one observer, and 200 cells were counted in each differential. Periods of observation continued until the temperature returned to the pre-treatment level. The periods of observation varied from 10 to 16 hours in duration. At the end of the period of observation treatment was continued until a total of 2.4 million units of penicillin-G had been given over an 8-day period.

Results. Of the 17 patients, 15 had definite febrile Jarisch-Herxheimer reactions (defined here as in temperature to 37.5°C or above, occurring within 10 hours after the start of treatment and returning to normal within 16 to 18 hours). The distribution of the temperature elevations was as follows:

Temperature height	No. of patients
37.8–38	3
38 –39	3
39 –40	8
40 –41	1

All temperatures were rectal and 37.5°C was taken as normal temperature.

In 10 of the 15 patients there was a definite leucocytosis occurring at the height or just before the height of the reaction, as judged by the temperature peak. The leucocytosis here is measured by the percentage change in total leucocyte count, from the base line count. No change of less than 40% in total count was considered significant. The average percentage change in the ten cases considered was 89%. The lowest change was 50% and the highest 113%. In the remaining 5 cases having Jarisch-Herxheimer reactions, a leucocytosis was noted but the percentage change was not above 40%. The variations in counts that occurred in these 5 patients are as follows: 15%, 20%, 34%, 15%, and 34%. The duration of the leucocytosis was transient, the

* Aided by a grant from the Syphilis Study Section, National Institute of Health, Bethesda, Maryland.

1 Willcox, R. R., *J. Royal Army Med. Corps*, 1923, **40**, 48.

2 Whitby and Britton, Disorders of the Blood, Blakiston and Co., Philadelphia, 1942, p. 457.

3 Wintrobe, Clinical Hematology, Lea and Febiger, Philadelphia, 1942, p. 123.

4 Downey, Handbook of Hematology, Paul Hoeber, New York, 1938, p. 675.

5 Spangler, R. H., *J. Lab. and Clin. Med.*, 1935, **20**, 733.

6 Sheldon and Heyman, Recent Advances in the Study of Venereal Diseases, 1948, p. 129.

7 Sheldon, W. H., personal communication.

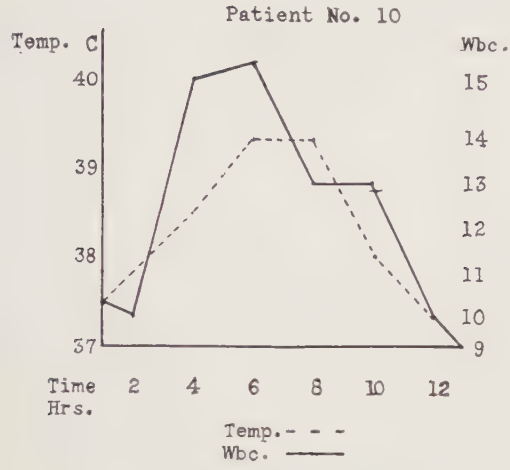

FIG. 1.

average duration being 4.4 hours. The longest period of duration was 8 hours, the shortest was 2 hours.

Fig. 1 indicates the type of response seen during the Jarisch-Herxheimer reaction.

Complete differential counts were done on 8 of the 15 patients every 2 hours during the Jarisch-Herxheimer reaction. The changes observed here were an increase in neutrophils with a lymphopenia. The average percentage increase in polymorphonuclear cells was 43.4% with a maximum change of 87% and a minimum change of 23%. The average percentage drop in lymphocytes was 55% with a maximum drop of 77% and a minimum drop of 39%. The maximum drop in lymphocytes occurred in the cases with the maximum increase in polymorphonuclear cells, and the minimum drop in lymphocytes occurred in the cases with the minimum increase in polymorphonuclear cells.

Discussion. The occurrence of the leucocytosis was at first felt to be caused by the fever associated with the Jarisch-Herxheimer reaction and not related to the mechanism of the reaction. The pattern of leucocyte response found in induced fever was found to be quite different;[8,9] the rise in leucocytes in induced fever usually occurred after the height of the fever and the leucocytosis continued for several hours after a return to normal temperature. In this study the leucocytosis usually occurred 2 to 4 hours before the temperature spike and returned to the base line count at the same time or just before the temperature. It therefore appeared that the mechanism causing the leucocytosis was the same mechanism as that causing the Jarisch-Herxheimer reaction. The obscurity of the mechanism involved in the production of the Jarisch-Herxheimer reaction prevents any conclusions as to the causative factors. However, it is interesting to note that with the leucocytosis there is a neutrophilia which corresponds in time with the acute polymorphonuclear infiltration around syphilitic lesions as described by Sheldon and Heyman.[6,7]

Summary. 1. In 10 of 15 patients having febrile Jarisch-Herxheimer reactions there was a definite leucocytosis occurring before or at the same time as the temperature spike. Some degree of leucocytosis occurred in the other 5 cases but was not considered significant.

2. The average percentage increase in leucocytes was 89%.

3. As shown by differential counts the increase in cells was predominantly in the polymorphonuclear series.

4. There was also a lymphopenia with an average percentage drop in total lymphocyte counts of 55%.

5. The causative factor in the leucocytosis and the causative factor of the Jarisch-Herxheimer reaction are felt to be the same.

[8] Bierman, W., and Fishberg, E. H., *J. Am. Med. Assn.*, 1934, **103**, 1354.

[9] Bierman, W., *Am. J. Med. Sci.*, 1934, **187**, 545.

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17259. A New Method of Flame Photometry.*

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An adequate review of the principles and background of flame photometry is given in the papers of Barnes *et al.*[1,2]

In order to accomplish accurate quantitative analysis by flame photometry, the element to be determined must be in solution in the form of a salt and this solution must in some manner be atomized into a flame of *constant thermal output*, so that a *constant amount* of the solution is introduced into this flame *per unit time*.

All previous workers have utilized an atomizer-burner system which contained a chamber through which the atomized vapor of the solution passed before entering the exciting flame, and all commercial models of flame photometers which have been marketed in this country and abroad are based on this principle.

After working with such a chamber atomizer-burner system for many months, we found it impossible to obtain consistently reproducible and accurate quantitative results to within one per cent. Moreover, we could not consistently introduce a constant amount of solution into a flame per unit time. This was due mainly to the fact that when an aqueous solution is atomized and passed through a chamber, the size of the droplet particles which enter the exciting flame is not uniform. This factor is more or less uncontrollable, because the particle size and the rate of change of size are dependent upon numerous and variable physical conditions, among which are: (a) temperature of the atomizer chamber; (b) viscosity of the solution being atomized; (c) surface tension of the solution being atomized, and (d) certain

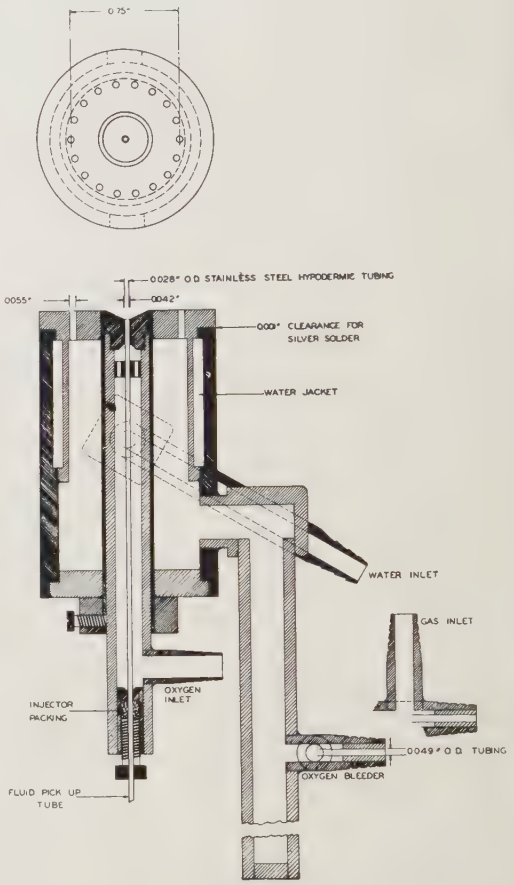

FIG. 1.
Diagram of Atomizer Burner System.

This system uses a mixture of illuminating or propane gas and oxygen for the thermal excitation of the atomized solutions. Atomization is accomplished by the use of oxygen under a constant pressure of 5 to 7 lbs., which enters the tube labeled oxygen-inlet and escapes at the narrow space below the fluid pick-up tube and the atomizer tip, producing a venturi effect, which accomplishes both the pick-up and atomization of the fluid to be analyzed. The fluid pick-up tube is immersed in a small glass beaker which contains the solution to be analyzed.

Approximately 0.75 ml of solution is atomized per minute, with a consumption of 11 cubic feet of oxygen per hour at 7 lbs. delivery pressure. To all solutions analyzed is added 0.04% STEROX SE (Monsanto Chemical Company), a non-ionic wetting agent, cation free, which overcomes the capillarity effects of the fluid pick-up tube.

* This work was partially supported by a grant from the Commonwealth Fund of New York.

1 Barnes, R. B., Richardson, D., Berry, J. W., and Hood, R. L., *Indust. and Eng. Chem., Anal. Ed.*, 1945, **17**, 605.

2 Berry, J. W., Chappell, D. G., and Barnes, R. G., *Indust. and Eng. Chem.*, 1946, **18**, 19.

TABLE I.

Deviations of Sodium and Potassium Determinations in Urine and Plasma by the Present Method of Flame Photometry from Sodium and Potassium Determinations by Chemical Methods.

Sodium					Potassium				
No.	Flame photometry, meq.	Gravimetric determin., meq.	Difference, meq.	Difference, %	No.	Flame photometry, meq.	Volumetric determin., meq.	Difference, meq.	Difference, %
1	147.1	143.9	+3.2	+2.2	1	3.83	3.74	+0.09	+2.4
2	144.9	143.7	+1.2	+0.8	2	3.40	3.29	+0.11	+3.3
3	143.8	144.7	—0.9	—0.6	3	4.30	4.40	—0.10	—2.3
4	145.8	143.7	+2.1	+1.5	4	4.76	4.67	+0.19	+1.9
5	112.0	111.8	+0.2	+0.2	5	4.48	4.38	+0.10	+2.3
6	146.8	144.8	+2.0	+1.4	6	4.70	4.64	+0.06	+1.3
7	135.2	137.8	—2.6	—1.9	7	4.46	4.48	—0.02	—0.4
8	140.5	143.3	—2.8	—2.0	8	4.42	4.41	+0.01	+0.2
9	142.5	140.0	+2.5	+1.8	9	5.59	5.61	—0.02	—0.4
10	133.7	135.5	—1.8	—1.3	10	4.65	4.82	—0.17	—3.5
11	138.8	140.0	—1.2	—0.8	11	4.85	4.73	+0.12	+2.5
12	135.5	136.2	—0.7	—0.5	12	4.68	4.54	+1.4	+3.1
13	91.0	88.7	+2.3	+2.6				Avg error 2.0%	
14	92.3	91.0	+1.3	+1.4				Range —3.5 to +3.3%	
15	91.0	90.7	+0.3	+0.3					
16	180.8	178.1	+2.7	+1.5					
17	180.0	178.8	+1.2	+0.7					
18	176.0	178.8	—2.8	—1.6					
19	142.0	143.1	—1.1	—0.8					
20	144.0	145.0	—1.0	—0.7					
21	139.0	138.3	+0.7	+0.5					
			Avg error 1.2%						
			Range —2.0 to +2.6%						

electrostatic effects due to variations of charge on the chamber walls.

The above conclusions were reached primarily from visual observations of the atomization of certain solutions containing fluorescein, under illumination with filtered ultraviolet light, under various standard laboratory conditions. For these reasons we decided to abandon the chamber and devise an atomizer which permitted the direct and immediate combustion of the atomized droplets.

Mention must also be made of the rather well-known spectroscopic phenomena of mutual excitation. This is illustrated by the production of more luminous energy per meq. of potassium in the presence of a large excess of sodium than from potassium in pure solution. This difficulty may be overcome by using as standards solutions containing a ratio of interfering elements, which only approximate that in the solution analyzed. For instance, in the case of Na and K, there is a wide plateau of mutual excitation from 18 Na: 1K to 56 Na: 1K, wherein no increase of excitation occurs. A similar compensation is made for the less frequently observed phenomenon of quenching.

We have found it unnecessary to use an internal standard method with this atomizer-burner system. Direct experiment showed no interferences, such as reported by Barnes *et al.*,[1,2] from the presence in the solutions of the common inorganic acids up to 2% concentration, or of urea or sucrose up to at least 4% concentration.

Fig. 1 is a schematic illustration of the atomizer-burner system developed in order to achieve this result, and thus overcome the above mentioned difficulties. With this apparatus we have been able to introduce a constant amount of the atomized solution per unit time into a flame of constant thermal out-

put. Several different glass filter and interference filter photometric devices have been successfully utilized with this burner-atomizer system. These include a simple barrier layer photocell-suspension type galvanometer system, vacuum phototube-electronic voltmeter, and a direct current operated photomultiplier tube-suspension galvanometer system.

Comparison of the results of sodium and potassium determinations in urine and plasma by standard chemical methods,[3,4] with the method of flame photometry described herein, are shown in Table I. We do not believe that this comparison reflects the true accuracy of this method of flame photometry, but represents at the present time the only acceptable means of evaluation.

The reproducibility, duplicability and recovery of added sodium and potassium to urine and plasma are of the order $\pm 0.5\%$. Further work is in progress for the determination of calcium, magnesium and other cations. Further data, as well as information as to the commercial availability of this instrument, will be available in a forthcoming publication.

Summary. A new type of atomizer-burner system is described for use in flame photometry which accomplishes consistently accurate quantitative chemical analysis of sodium, potassium, and other cations.

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[3] Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, **93**, 171.

[4] Folch, J., and Lauren, M., *J. Biol. Chem.*, 1947, **169**, 539.

17260. Inhibition by Cyanide of Serum Alkaline Phosphatase in Normal Man, Obstructive Jaundice and Skeletal Disorders.

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It has been the general experience that marked increases in serum alkaline phosphatase activity occur consistently in only 2 disease categories in man: 1. in skeletal disorders associated with active, widespread proliferation of bone or cartilage, 2. in diseases of the liver or biliary tract with obstruction of the intra- or extrahepatic biliary channels.[1] There is general agreement that the high serum alkaline phosphatase levels observed in skeletal disorders are the result of augmented production of the enzyme by osteoblasts, which are known to be a prolific source. Opinion is divided as to the origin of the increased serum alkaline phosphatase in biliary tract obstruction. It would seem reasonable to suppose that since alkaline phosphatase is excreted in the bile and (being a large protein molecule) does not escape through intact glomeruli in man, retention of the enzyme in the plasma occurs when the excretory biliary channels are obstructed. There is, however, the possibility that the increased enzyme appearing in the plasma is of hepatic origin, and perhaps a different alkaline phosphatase from that responsible for most of the enzyme activity exhibited by the plasma of normal subjects and of those with skeletal diseases.

Drill and coworkers [2,3] reported that sodium cyanide in concentrations to 0.1M only slightly inhibited the serum alkaline phosphatase activity of normal dogs and man whereas the elevated levels in hepatic damage were markedly reduced, to approximately normal levels but not below. Their data were interpreted as indicating that the alkaline phosphatase appearing in the serum of dogs and man with hepatic damage is, for the most part,

[1] Gutman, A. B., Olson, K. B., Gutman, E. B., and Flood, C. A., *J. Clin. Invest.*, 1940, **19**, 129.

[2] Drill, V. A., Annegers, J. H., and Ivy, A. C., *J. Biol. Chem.*, 1944, **152**, 339.

[3] Drill, V. A., and Riggs, D. S., *J. Biol. Chem.*, 1946, **162**, 21.

TABLE I.
Effect of NaCN on Serum Alkaline Phosphatase Activity.

	Mols NaCN added to substrate					
	0	0.001	0.005	0.01	0.05	% initial activity remaining
	(Alkaline phosphatase, Bodansky units/100 cc serum)					
Normal human subjects						
1. Adult	3.0	2.1	0.8	0.5	—	16.7
2 "	2.7	1.6	0.6	0.3	0.4	11.1
3. "	2.1	1.8	0.8	0.6	0.5	23.8
4. "	2.0	1.5	0.5	0.3	0.4	20.0
5. Infants (pooled)	7.9	5.4	1.1	0.6	0.7	7.6
6. " "	6.0	—	1.0	0.9	1.0	16.7
Obstructive jaundice						
7. Biliary cirrhosis	46.7	28.8	11.9	3.2	—	6.9
8. Choledocholithiasis	27.2	—	7.4	2.0	1.4	5.1
9. Unclassified	22.5	—	4.7	1.4	1.3	5.8
10. ? Ca gall bladder	17.3	10.8	3.9	1.3	—	7.5
11. Cholangiolitic cirrhosis, marked parenchymal damage	16.2	—	2.7	1.2	0.9	5.6
12. Ca head of pancreas, cirrhosis	13.2	—	3.8	1.1	1.0	7.6
Skeletal disorders						
13. Paget's disease	75.9	—	11.3	3.3	2.4	3.2
14. Metastatic prostatic carcinoma	38.6	24.6	9.0	2.0	—	5.2
15. " " "	27.3	18.8	6.3	1.7	0.9	3.3
16. " " "	27.0	17.2	5.5	1.5	—	5.5
17. " " "	25.7	—	5.7	1.8	0.9	3.5
18. " " "	20.3	—	5.4	1.9	0.8	3.9
19. " " "	19.9	—	3.9	1.8	1.4	7.0
20. Paget's disease	9.1	—	2.4	0.6	0.6	6.6
21. Metastatic prostatic carcinoma	7.2	—	2.1	1.0	1.1	13.9

different from that normally present. We have repeated and extended these studies to include the effects of sodium cyanide on increased alkaline phosphatase levels in diseases of bone.

Methods. Serum alkaline phosphatase was determined by the Bodansky method.[4] A stock solution of 0.1M NaCN in buffered β-glycerophosphate solution was prepared and the pH of this solution adjusted to 9.2. Appropriate amounts of this stock solution were added to β-glycerophosphate substrate to make the desired cyanide concentration, the pH again checked, and the cyanide-substrate mixture then added to the serum just prior to incubation. Preliminary tests indicated that these concentrations of cyanide had no significant effect on the development of color by the inorganic phosphate standard.

Results. Four samples of normal adult human sera and 2 of pooled infants sera were examined and in each instance marked inhibition by cyanide was observed (Table I) The degree of inhibition increased with increasing concentrations of cyanide, at least up to 0.01M, although there was always some residual enzyme activity at the end of 1 hour incubation, varying from 0.4-1.0 Bodansky units % or 7.6-23.8% of the initial activity. The data indicate that most of the alkaline phosphatase present in normal human serum is inhibited by cyanide. These results are not in complete disagreement with those of Drill and Riggs[3] since examination of their data reveals inhibition greater than 50% in 2 of their 6 normal sera, and in several normal sera preincubation with NaCN disclosed inhibition of the order we obtained.

The data in 6 cases of obstructive jaundice with elevated serum alkaline phosphatse (Table I) confirm the previous finding[3] of marked inhibition by cyanide, to 5.1-7.6% of the initial activity in our series. In most instances we found the residual enzyme activity somewhat below the normal minimum of 1.5 Bodansky units %; it was higher only in

[4] Bodansky, A., *J. Biol. Chem.*, 1933, **101**, 93.

1 serum with very marked initial activity.

In 9 patients with elevated serum alkaline phosphatase due to increased bone formation (in which the excess enzyme appearing in the serum is doubtless of osseous origin) the inhibitory effects of cyanide were indistinguishable from those observed in obstructive jaundice (Table I). The residual activity, 0.6-2.4 Bodansky units % was consistently less than the normal minimum except in the 2 cases with very high initial levels. The % activity persisting, 3.2-13.9% of the original values, was of the same order as observed in obstructive jaundice. A somewhat higher percentage of the original activity, approximately that observed in normal sera after inhibition by cyanide, persisted when initial levels were only moderately elevated.

Our data disclose no essential differences in the inhibiting effect of cyanide in all 3 categories; most of the serum alkaline phosphatase activity present normally, in obstructive jaundice and in skeletal disorders alike was inhibited by cyanide. Moreover, since 100% inhibition of an enzyme is infrequently achieved, the residual enzyme activity, low as it was in most sera, probably represents maximal values for cyanide-insensitive alkaline phosphatases present in serum. That we apparently did not obtain complete inhibition of cyanide-sensitive phosphatases is indicated by the persistence of greater residual activity in sera with higher initial levels.

Discussion. The data obtained appear to throw light upon 2 obscure points, 1. the origin of the alkaline phosphatases in the plasma of normal human adults*, 2. the origin and causal mechanism of the increased serum alkaline phosphatase in obstruction of the extra- or intrahepatic biliary tract.

Cloetens[5] distinguished 2 classes of alkaline phosphatase: Phosphatase I, inactive in the absence of Mg^{++}, markedly activated by appropriate concentrations of Mg^{++}, not inhibited by cyanide when so activated; phosphatase II, active without addition of Mg^{++} and only little affected by addition of Mg^{++} but markedly inhibited by cyanide. Cloetens[5] found that phosphatase II, cyanide-sensitive, accounted for over 90% of the total alkaline phosphatase activity of bone (which we have confirmed in unpublished experiments; see also O. Bodansky[6]), 96-99% of that of the serum of 3 rachitic dogs, and 97% of that of the serum of 1 dog with liver disease. Liver tissue phosphatase, in contrast, was usually more than 60% phosphatase I, cyanide-resistant.[5†] In our experiments,[‡] inhibition by cyanide of 76.2-92.4% of the alkaline phosphatase activity of normal human sera indicates that most of this activity is due to a phosphatase II, like bone phosphatase but unlike most liver phosphatases. Belfanti *et al.*,[7] using oxalate inhibition as a criterion, also found that the alkaline phosphatase of normal serum resembled bone phosphatase but differed from liver phosphatase. The largest proportion of the alkaline phosphatases present in normal human serum would therefore appear from this chemical evidence to be indistinguishable by available methods from bone phosphatase; a small proportion, cyanide-resistant, is probably not of osseous but of other (undetermined) origin. This interpretation is consistent with observations in hepatectomized and eviscerated dogs[8,9] indicating that serum alkaline phosphatase levels

* It is generally accepted that the increased serum alkaline phosphatase of growing children, like that of patients with bone or cartilage proliferation due to skeletal disorders, is due to increased osteoblastic activity.

[5] Cloetens, R., *Enzymologia*, 1939, **6**, 46.

[6] Bodansky, O., *J. Biol. Chem.*, 1949, **179**, 81.

† Presumably, much of the cyanide-sensitive phosphatase II found in liver is serum phosphatase in the blood and bile present in liver tissue preparations.

‡ We did not examine the effects of addition of magnesium ions (see Drill and Riggs[3]) because the presence of magnesium in serum makes interpretation difficult. Moreover, Cloetens worked largely with dialysed tissue extracts and the differences he noted in the effects of addition of Mg^{++} probably reflect differences in the ease with which Mg ion is removed by dialysis from the various alkaline phosphatases of different organs. This factor does not enter into our experiments.

[7] Belfanti, S., Contardi, A., and Ercoli, A., *Biochem. J.*, 1935, **29**, 1491.

[8] Armstrong, A. R., and Banting, F. G., *Can. Med. Assn. J.*, 1935, **33**, 243.

are maintained under these conditions and that the enzyme hence cannot be of hepatic origin.

Our data indicate that the increased serum alkaline phosphatase in obstructive jaundice, as in skeletal diseases, is phosphatase II (cyanide-sensitive) and therefore presumably not of hepatic cell origin. The results are compatible with the view that elevation of serum alkaline phosphatase in extra- or intrahepatic biliary tract obstruction is due to retention of serum alkaline phosphatase, largely of osseous origin. There is a great deal of clinical evidence for this view which was summarized elsewhere.[1]

The marked difference in the effects of cyanide on serum alkaline phosphatase and on liver tissue alkaline phosphatase indicates definite differences between these enzymes. However, the similarity between serum alkaline phosphatase and bone phosphatase with respect to cyanide does not prove their identity; all that can be said is that in this and other respects no significant difference is apparent. Absolute proof of identity of 2 enzymes from different sources is not possible at this time.

It may seem surprising that with so many alkaline phosphatases present in so many organs, the enzyme in the plasma should be so preponderantly of osseous origin. However, not only is the number of osteoblasts in the body very large and their alkaline phosphatase production great, but the secretion of the enzyme is extracellular for production of bone at the cell surface. In most cells, phosphatases operate intracellularly, often apparently within the confines of the nucleus to judge from histochemical evidence, and probably never reach the extracellular fluids.

Summary. Cyanide markedly inhibits the serum alkaline phosphatase of normal human subjects and the increased levels of patients with obstructive jaundice and skeletal diseases; no essenital differences were observed in these 3 categories. The evidence is consistent with the view that in all 3 categories the largest proportion of serum alkaline phosphatase is of osseous origin since bone phosphatase is inhibited by cyanide (Cloetens' phosphatase II) and liver phosphatases are, for the most part, cyanide-insensitive (Cloetens' phosphatase I). The data indicate that the rise in serum alkaline phosphatase in obstructive jaundice cannot be of hepatic origin but they are compatible with retention of serum alkaline phosphatase due to obstruction of the excretory biliary channels.

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[9] Maddock, S., Schmidt, G., and Thannhauser, S. J., *Fed. Proc.*, 1942, **1**, 181.

17261. Intracellular Distribution of Vitamin B_6 in Rat and Mouse Livers and Induced Rat Liver Tumors.*

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In studies[1-4] from this laboratory the intracellular distribution of riboflavin was determined in normal rat livers, in the livers of rats fed various aminoazo dyes, and in liver tumors induced by 4-dimethylaminoazobenzene. Riboflavin is of particular interest since

* This work was supported in part by grants from the National Cancer Institute, United States Public Health Service, and the Jane Coffin Childs Memorial Fund for Medical Research.

† Predoctorate Research Fellow, National Cancer Institute.

[1] Price, J. M., Miller, E. C., and Miller, J. A., *J. Biol. Chem.*, 1948, **173**, 345.

[2] Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research*, 1949, **9**, in press.

[3] Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., manuscript in preparation.

[4] Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research*, 1949, **9**, 96.

high dietary levels of this vitamin delay the induction of neoplasms by certain of the aminoazo dyes.[5] Furthermore, ingestion of any one of the various dyes lowers the level of riboflavin in the liver to a degree that is roughly proportional to its carcinogenic activity.[6,7] While the ingestion of 4-dimethylaminoazobenzene also decreases the level of vitamin B_6 in the liver this vitamin differs from riboflavin in that the various dietary changes which enhance or lower the carcinogenic activity of this dye do not alter the level of hepatic vitamin B_6 in a significant fashion.[7] Hence it was desirable to compare the intracellular distribution of vitamin B_6 with that of riboflavin in the livers of rats before and after the administration of 4-dimethylaminoazobenzene as well as in hepatic tumors induced by this dye. Similar data for the liver of the mouse, a species relatively resistant to the carcinogenic action of this dye,[5,8] are also presented.

Methods. The animals were fed a semisynthetic diet (diet 3[7]) containing 1.2 mg of riboflavin per kg. Male albino rats‡ were fed this basal diet with or without 0.06% 4-dimethylaminoazobenzene for 4 weeks. Female albino mice§ were fed the same diets but for 4 months. The animals were killed with ether and the livers perfused *in situ* with isotonic saline. The perfusion and all subsequent steps prior to the analytical procedures were carried out at 0 to 5°. The rat liver tumors were obtained from rats fed the basal diet plus the dye for 4 to 5 months. Nine mouse livers were pooled for fractionation No. 1 and 7 livers were combined for fractionation no. 2. Each fractionation of rat liver was made on pools from 2 (fractionations no. 3 and 5) or 3 (fractionations no. 4 and 6) animals. Small tumors which were grossly non-necrotic were pooled from 9 and 20 rats for fractionations no. 7 and 8, respectively. The selection of the tumors, which were obtained from non-perfused livers, was made as previously described.[4] The pooled tumors and livers were forced through a plastic tissue mincer and homogenized in 0.88 *M* sucrose solution as previously described.[1]

Differential centrifugation was used as described previously[1,2,4] to prepare the nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions. The nuclear and large granule fractions were washed twice; the small granules were sedimented at 24,000 x *g* (at center of tube) for 3 hours and were not washed. In experiments where the small granules were washed the nuclear and large granule fractions were sedimented in the usual manner, but were washed only once. The supernatant fluid and washing from the large granules were combined and centrifuged for 36 minutes at 120,000 x *g* (at center of tube) in an air-driven ultracentrifuge. The small granules were then resuspended in the sucrose solution and, after aliquots had been taken for analysis, were brought to the volume in which they had previously been suspended. After recentrifugation under identical conditions the sediment was suspended in the sucrose solution for analysis.

Vitamin B_6 was determined with *Saccharomyces carlsbergensis* by the method of Atkin *et al.*[9] This yeast responds equally to the 3 known forms of vitamin B_6.[10] The intracellular distributions of protein, nucleic acids, and riboflavin were also determined as previously described.[1] The intracellular distributions of these constituents in the fractionations of the rat tissues agreed well with those previously published.[1,4]

Results. From Table I it is evident that most of the vitamin B_6 was in the large granules and the supernatant fluid in all of the tissues studied. The large granules con-

[5] Rusch, H. P., Baumann, C. A., Miller, J. A., and Kline, B. E., in Moulton, F. R., A.A.A.S. research conference on cancer, Washington, 1945, 267.

[6] Griffin, A. C., and Baumann, C. A., *Arch. Biochem.*, 1946, **11**, 467.

[7] Miller, E. C., Miller, J. A., Kline, B. E., and Rusch, H. P., *J. Exp. Med.*, 1948, **88**, 89.

[8] Kirby, A. H. M., *Cancer Research*, 1945, **5**, 683.

‡ Holtzman Rat Company, Madison, Wis.

§ A. Sutter, Springfield, Mo.

[9] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

[10] Snell, E. E., *Physiol. Rev.*, 1948, **28**, 255.

TABLE I.
Intracellular Distribution of Vitamin B_6 in Liver and Liver Tumor.

Fraction	Mouse livers		Rat livers				Rat liver tumors	
	Basal diet	Basal diet + dye	Basal diet		Basal diet + dye			
	Fractionation No.							
	1	2	3	4	5	6	7	8
	μg of vitamin B_6 per g of fresh tissue							
Whole homogenate	6.0	4.5	6.4	6.4	4.0	4.7	1.8	1.2
Nuclei	0.5	0.5	0.4	0.2	0.3	0.2	0.2	0.1
Large granules	2.0	1.4	2.3	2.9	1.6	1.9	0.5	0.4
Small "	0.3	0.2	0.3	0.4	0.2	0.2	0.2	0.1
Supernatant fluid	3.0	2.2	3.0	3.1	1.8	2.1	1.2	0.7
Recovery	5.8	4.3	6.0	6.6	3.9	4.4	2.1	1.3
	μg of vitamin B_6 per g of protein							
Whole homogenate	46	34	53	50	37	40	15	10
Nuclei	22	18	26	14	18	11	5	2
Large granules	57	57	60	73	60	58	46	29
Small "	19	16	18	21	17	13	12	9
Supernatant fluid	60	36	64	62	40	41	26	14

tained 28 to 45% and the supernatant fluid 45 to 64% of the total amount of vitamin B_6. The ratio of vitamin B_6 to protein was higher in the large granule and supernatant fluid fractions, and lower in the nuclear and small granule fractions than it was in the whole homogenate. When 4-dimethylaminoazobenzene was included in the diets of either rats (fractionations no. 5 and 6) or mice (fractionation no. 2) the amount of vitamin B_6 in the large granule and supernatant fluid fractions was considerably reduced. Since the protein content was also reduced in the large granule fraction[1,2] the ratio of vitamin to protein was unchanged in this fraction. On the other hand this ratio was decreased in the supernatant fluid since the protein content of this fraction remained unchanged.[1,2] In the liver tumor tissue the level of vitamin B_6 in the large granules and supernatant fluid was even lower than in the livers of rats fed the dye, and the ratio of the vitamin to protein was also much lower.

The small granules isolated from the livers of rats fed the basal diet after centrifuging at 120,000 x *g* for 36 minutes were entirely comparable in their contents of pentosenucleic acid, riboflavin and vitamin B_6 to those isolated from similar livers at 24,000 x *g* for 3 hours. A single washing reduced the level of vitamin B_6 in these particles by 50 to 80%. The pentosenucleic acid was reduced by 13 to 28% and in a single experiment no detectable loss of riboflavin occurred on washing. These changes are probably attributable in part to the removal of some residual supernatant fluid from the small granules.

Discussion. As in the case of riboflavin[1-4] the vitamin B_6 was found chiefly in the large granules and supernatant fluid in all the tissues studied. The nuclear fraction of normal rat liver contained 3 to 6% of the total vitamin B_6 whereas this fraction generally contains 5 to 9% of the total riboflavin. The greatest differences in the distribution of the two vitamins was found in the small granules. The washed small granules contained only 0.8 to 1.9% of the total vitamin B_6 but still contained 16% of the total riboflavin. It is probable that the values for the vitamin B_6 and riboflavin contents of the nuclear fraction are too high, since this fraction is contaminated with some whole cells and large granules.[1,11]

Claude[12] stated that he found most of the transaminase activity of rat and guinea pig liver in the supernatant fluid. The transa-

[11] Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, 1948, **172**, 619.

[12] Claude, A., in Moulton, F. R., A.A.A.S. research conference on cancer, Washington, 1945, 223.

minase activity was relatively low in the large granules and was completely absent from the microsome or small granule fraction. Subsequent to these observations it was found that vitamin B_6 is an integral part of this enzyme system.[13] Thus the data presented above support those of Claude since the small granules contain no transaminase activity and very little vitamin B_6 while the parts of the liver cell that contain transaminase activity are particularly rich in vitamin B_6. No information is available on the presence or distribution in these tissues of other enzymes which contain vitamin B_6.

The hepatic carcinogen 4-dimethylaminoazobenzene reduced the level of riboflavin[1,2] and vitamin B_6 in the large granules and supernatant fluid, but in both cases the reduction of protein in the large granules paralleled the decrease in vitamin content. This is probably the result of a reduction of the number of large granules in the liver cells.[3]

Summary. The intracellular distribution of vitamin B_6 in rat liver tumors induced by 4-dimethylaminoazobenzene and in the livers of rats and mice before and after administration of the azo dye was determined after differential centrifugation of the tissue homogenates in hypertonic sucrose solution.

In all cases vitamin B_6 was concentrated in the washed large granules or mitochondria (28 to 45% of total) and in the supernatant fluids (45 to 64% of total). The washed nuclear fractions contained 3 to 11% of the total vitamin B_6 present. The unwashed small granules or microsomes contained 4 to 8% of the total vitamin B_6; in the case of normal rat liver one washing reduced the level to about 1½%.

The ingestion of the azo dye reduced the levels of the vitamin in the large granule and supernatant fluid fractions of the livers in each species. The decrease in protein content of the large granules paralleled the decrease in vitamin B_6 content. The large granule and supernatant fluid fractions of the liver tumors contained even less vitamin B_6 than was found in the corresponding fractions from the livers of rats fed the dye and the ratio of the vitamin to protein was also much lower.

[13] Wynne, A. M., in *Ann. Rev. of Biochem.*, 1946, **15**, 58.

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17262. Healing of Tuberculous Pulmonary Cavities by Means of Skin Grafts.

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Giant tuberculous pulmonary cavities continue to present one of the most complex problems in the surgical treatment of pulmonary tuberculosis. Various types of collapse therapy have resulted in a high percentage of failures. Intracavitary drainage in tension or blocked cavities, combined with collapse therapy, has greatly increased the number of good results.

Intracavitary drainage without collapse therapy provides relief from toxicity but is of little value in cavity closure. In advanced pulmonary disease with multiple cavitation, collapse therapy is contraindicated.

We wish to present a somewhat different point of view. Let us consider that a tuberculous pulmonary cavity is a chronic lung abscess: The lung surrounding a chronic nonspecific lung abscess shows little disease. When such an abscess is drained, the surrounding lung tissues fill in the defect occupied by the abscess. In a tuberculous lung abscess, the surrounding lung tissues are usually diseased and cannot expand to fill in the space occupied by the abscess cavity. Drainage is of cleansing value in pulmonary tuberculous cavities, as in other types of pulmonary abscesses, but cavity obliteration does not occur without col-

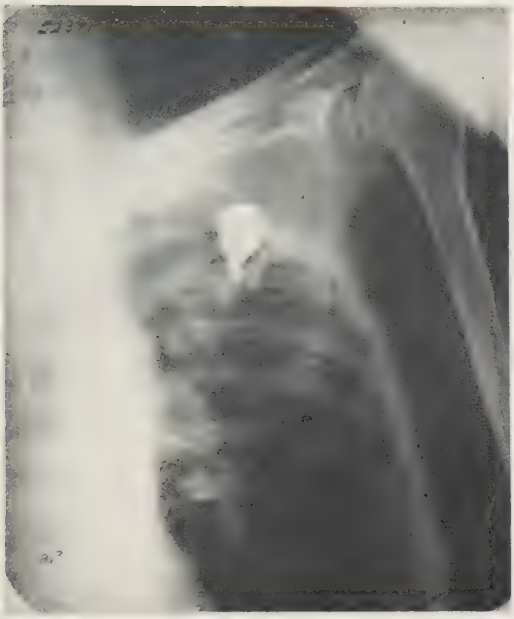

FIG. 1.
Five months following cavernostomy and repeated skin grafts the lipiodol is lying on the skin lining the former cavity which is continuous with the skin of the chest wall and does not enter the bronchial tree.

lapse therapy.

At the Grace Dart Home Hospital large cavities have been treated by intracavitary drainage. The residual cavity space has been opened into by cavernostomy, carried out through the sinus tract of the drainage tube. The exposed cavity walls and floor have been covered with split-thickness skin grafts or pinch grafts. Gradually the boundaries of the cavity are lined with skin which grows out to meet the skin on the surface of the chest wall. The cavity thus becomes obliterated, leaving a defect in the chest wall.

The first case selected was a 52-year-old male with far-advanced bilateral pulmonary

FIG. 2.
Section taken through wall of skin grafted cavity. Note lung covered by a layer of skin with proliferation of fibrous tissue and compressed pulmonary alveoli.

disease with giant bilateral apical cavities. On February 4, 1948, an anterior stage thoracoplasty was done on the left side. On May 28, 1948, intracavitary drainage was established in the left apical cavity. The poor general condition of the patient prevented further collapse therapy. On September 22, 1948, the left apical cavity was entered into through the drainage tract. Cavernostomy was done, and the depths of the cavity were lined with a split-thickness skin graft. This was repeated on October 20, 1948, November 17, 1948 and on February 9, 1949; on the last occasion pinch grafts were used. In all instances mentioned, at least a 75% "take" of the graft was obtained. The cavity on the left side diminished and gradually was covered with skin; the bronchial openings, which at first were large, gradually closed. At autopsy, May 8, 1949, there was no evidence of an open bronchus leading to the skin surface. The surface skin on the chest wall showed a depression about 1½″ in diameter which entered into the lung. The cavity seemed to be completely covered by skin and was entirely exteriorized. The cavity on the right side remained unchanged. Two other cases have been treated. In both skin grafting has been successful and the cavities seem to be healing.

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17263. Effect of Alloxan in Rabbits with Temporary Occlusion of the Arteries to the Pancreas.*

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Numerous investigations have shown that the injection of alloxan into various animals causes destruction of the pancreatic islets of Langerhans with the production of diabetes. After the injection of a diabetogenic dose of alloxan intravenously into a rabbit hyperglycemia appears which reaches its peak within 2 hours. This in turn is followed in 2 to 9 hours by profound hypoglycemia frequently with convulsions. Finally within 24 to 48 hours diabetes develops and although most agree that the diabetes results from the destruction of the islets of Langerhans, the initial hyperglycemic phase and especially the hypoglycemic phase have caused much speculation and disagreement.

Although diabetes usually is considered to result from an action of the drug on the pancreatic islets, Jimenez-Diaz[1] reported that clamping the renal blood vessels immediately before the injection of alloxan prevents the development of diabetes. Others[2] have not been able to confirm this.

In the present investigation alloxan was injected into rabbits with the blood supply to the pancreas occluded temporarily in order (1) to confirm that alloxan diabetes is pancreatic in origin, and (2) to determine if the hypoglycemia is dependent upon alloxan producing histological changes in the islets of Langerhans.

Materials and Methods. Male albino rabbits weighing 2.0 to 2.7 kg were used and each was fasted for 16 to 18 hours before an experiment. Anesthesia was obtained by 18 to 20 mg/kg of 5% sodium pentobarbital intravenously supplemented by ether given by open mask as required. A transverse incision was made in the left upper quadrant of the abdomen extending quite far laterally. The celiac axis and the superior mesenteric artery were exposed and clamped close to the abdominal

* This work was aided by grants from the American Cyanamid Co., and the Diabetic Fund.

1 Jimenez-Diaz, G., Grande-Covian, F., and DeOya, J. C., *Nature*, 1946, **158**, 589.

2 Martinez, C., Gitter, S., and Covian, M. R., *Rev. Soc. argent de biol.*, 1947, **223**, 81.

aorta with rubber-tipped bulldog clamps. Each animal was then given 150 mg/kg of 5% alloxan (Eastman Kodak) into an ear vein during a 2 minute period and the clamps were left in place for a further period of 8 minutes. The clamps were then removed and the incision closed. Blood samples were obtained immediately before the alloxan injection and at frequent intervals thereafter, and the blood sugar was determined by the Folin and Malmros micromethod.[3]

The animals were sacrificed by an intravenous injection of sodium pentobarbital. A part of the pancreas was removed immediately and fixed in Bouin's fluid. The remainder of the pancreas and portions of other organs were fixed in Helly's fluid. Gomori's chrome alum hematoxylin stain[4] was used to differentiate beta and alpha cells in the islets of Langerhans, and hematoxylin and eosin was used on the other tissues.

Preliminary Experiments. In some preliminary experiments the celiac artery alone was clamped. Of 8 such animals given alloxan 2 developed severe diabetes at 24 hours with typical destruction of the pancreatic islets. Two others developed mild, transient, delayed hyperglycemia after 6 days but when studied histologically at 19 and 21 days no abnormalities were detected in the pancreas. The remaining 4 failed to show hyperglycemia in 19 to 37 days of study and on histological examination the pancreas appeared normal. Of the 6 animals which failed to develop typical diabetes 5 had shown definite early hypoglycemia with convulsions in 3 cases.

When India ink (20 cc of a 1 to 5 dilution) was injected into an ear vein with only the celiac artery clamped small quantities of the ink were detected in the pancreas indicating that the blood supply was not completely occluded. However, when both the celiac and mesenteric arteries were clamped and India ink injected no ink could be detected with certainty in the pancreas of 2 animals while in a 3rd only very small quantities were present. Control sections of the liver and kidney

TABLE I.

Effect of Alloxan on Blood Sugar of Rabbits with Celiac and Superior Mesenteric Arteries Occluded.

Rabbit No.	Blood sugar in mg/100 cc																		Notes
	Control†	Min.		Hr								Days							
	0	20	40	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	12-15	
1	127	177	152	142	133	87	124	106	97				70	137		93	104		99	105	Sacrificed 15 days
2	114	142	122	122	118	92	94	84	92				124	116		135	121		127	118	" 15 "
3	103	87	87	103	84	66	47*	65	65				96	108	114						" 3 "
4	114	127	137	153	148	139	87	70	57		43*		124	116	122						" 3 "
5	84	92	89	148	171	131	81	41*	50				159	—	139	171	258	230			" 6 "
6	94	93	84	87	75	45	34*						59	118							Died 2 "
7	101	109	114	109	150	101	88	73	37*				79	129	85				116	109	Sacrificed 12 "
8	99	120	128	131	114	112	93	82	69				96	105	92				108	116	" 12 "
9	89	81	85	90	120	127	105	106		62	52	46	106	116	112		108				" 5 "
10	103	81	101	112	135	118	97	68		59	60		175	156	153		123				" 5 "

* Hypoglycemic reaction.
† After induction of anesthesia, immediately before alloxan injection.

[3] Folin, O., and Malmros, H. J., *J. Biol. Chem.*, 1929, **83**, 115.

[4] Gomori, G., *Am. J. Path.*, 1941, **17**, 395.

showed large quantities of the ink.

In control animals with both the celiac and superior mesenteric arteries occluded but no alloxan or ink injected the blood sugar remained within a normal range.

Results. Table I shows the blood sugar of 10 rabbits given alloxan just after clamping the celiac and superior mesenteric arteries.

An initial hyperglycemic phase usually seen after the injection of alloxan in normal animals was present but not striking in most of these experiments.

Six of the 10 rabbits developed severe hypoglycemia within 3 to 9 hours after alloxan with blood sugar values of 34 to 47 mg/100 cc and 2 others had blood sugar values of 59 and 69 respectively. The remaining 2 failed to develop hypoglycemia by 6 hours when they were fed and the possibility exists that hypoglycemia might have occurred had they been fasted longer.

Nine of the 10 animals failed to develop diabetes. Eight were sacrificed in 3 to 15 days and one rabbit, which had unusually prolonged hypoglycemia, died of an undetermined cause 2 days after the injection with a normal blood sugar. In the remaining rabbit (No. 5) the blood sugar was normal for 3 days, reached 171 mg on the 4th day and 258 and 230 respectively, on the 5th and 6th day at which time the animal was sacrificed. Such a delayed onset of hyperglycemia occurs occasionally in unoperated rabbits given alloxan but usually definite hyperglycemia develops within 24 to 48 hours.

For histological examination 2 animals were sacrificed at 3 days, 3 at 5 days, 2 at 12 days and 2 at 15 days. No significant findings were noted in the liver, spleen, adrenals or kidneys in any of the animals. Seven rabbits (Nos. 2, 3, 6, 7, 8, 9, 10) showed no definite histological changes in the pancreas. The tissues of one animal (No. 1) were inadvertently destroyed. In the remaining 2 (Nos. 4 and 5), mild alterations in the pancreas were found.

In rabbit No. 5 which developed delayed hypoglycemia the islets showed (1) an apparent reduction in the total number of beta cells, some islets consisting solely of alpha cells; (2) a definite variation in size of the nuclei of beta cells, with some large hyperchromatic forms; (3) a total of 4 mitotic figures in beta cells found in examination of 2 different sections; and (4) a pallor of the cytoplasm of the beta cells, suggesting degranulation but without definite vacuoles. Rabbit 4, which had a normal blood sugar and appeared well when sacrificed 3 days after alloxan, showed the same type of histological changes but to a considerably less degree.

It should be pointed out that the changes described above are in marked contrast to the massive necrosis of beta cells which usually follows a diabetogenic dose of alloxan. The findings do suggest a mild injury of the beta cells with evidence of regeneration.

Discussion. These experiments strongly suggest that alloxan diabetes is pancreatic in origin for we have not observed definite diabetes when the pancreatic blood supply was interrupted sufficiently to prevent detectable changes in the islets of Langerhans.

Although very mild, transitory, delayed hyperglycemia was observed in 2 rabbits with only the celiac artery occluded and no islet changes were seen, which appears contradictory, it must be emphasized that these animals were sacrificed at 19 and 21 days after the administration of alloxan and it is well known that slight pancreatic islet damage observed in the first 2 or 3 days after alloxan injection may not be visible several days later. Since very small amounts of India ink were present in the pancreas of 1 of 3 animals with both celiac and superior mesenteric arteries occluded, and since 1 rabbit developed delayed hyperglycemia accompanied by slight but definite pancreatic changes, it is not certain that clamping these 2 vessels always eliminates completely the blood supply to the pancreas.

From these experiments it is impossible to say that no alloxan reached the pancreas and that the hypoglycemic phase was extrapancreatic in origin. However, hypoglycemia was observed to follow the injection of alloxan without the subsequent development of diabetes and without detectable changes in the islets of Langerhans even in 2 animals whose pancreas was examined at 2 and 3 days when alloxan damage to the islets should be severe.

These experiments suggest that the hypo-

glycemic phase is either extrapancreatic in origin as first suggested by Houssay[5] or that insulin is released from the islets by the action of a small quantity of alloxan insufficient to produce detectable histological changes.

After these studies had been completed we learned that Carrasco-Formiguera[6] had conducted experiments in 4 dogs in which alloxan, in dosage of 75 mg/kg, was injected into an intramesenteric vein ". . . while the pancreas was totally excluded from the circulation by clamping all its visible channels of irrigation, previous to, through 6 minutes after the said injection . . .". All 4 dogs developed profound hypoglycemia and in the 2 animals whose tissues were examined histologically the pancreas was said to be normal but the liver showed extremely severe lesions. Carrasco-Formiguera concluded from these experiments that the probable cause of alloxan hypoglycemia was liver damage. However, in our rabbits with occluded pancreatic circulation the alloxan was injected into an ear vein instead of into the portal system and hypoglycemia developed without detectable histological changes in the liver.

Summary. Diabetes was prevented in 9 of 10 rabbits given alloxan by temporary occlusion of the celiac and superior mesenteric arteries. The remaining animal developed moderate, delayed diabetes with mild histological changes in the islets of Langerhans.

Severe hypoglycemia was observed in several of the rabbits which failed to develop diabetes and which failed to show any histological change in the islets of Langerhans or the liver when examined at 2 to 15 days.

These experiments indicate that alloxan diabetes is pancreatic in origin but suggest that the hypoglycemic phase is either extrapancreatic in origin or may be produced by the action of very small quantities of alloxan insufficient to produce detectable changes in the islets of Langerhans.

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5 Houssay, B. A., Orias, O., and Sara, I., *Science*, 1945, **102**, 197.

6 Carrasco-Formiguera, R., *Arch. Biol. Pat. (Univ. de Los Andes)*, 1948, **1**, 111.

17264. Effect of Uric Acid in Glutathione-Deficient Rabbits.*

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The production of diabetes by the administration of alloxan or chemically related compounds, dialuric acid or alloxantin, has provoked considerable speculation whether these may occur in the body as normal or abnormal metabolites in sufficient concentration to damage the islets of Langerhans. Since alloxan can be obtained by the reduction of uric acid *in vitro,* Lazarow[1] suggests that an abnormal purine metabolism may possibly produce alloxan or an alloxan-like compound.

The injection of alloxan produces a profound decrease in blood reduced glutathione in rabbits[2] and rats[3] and the administration of glutathione prior to the injection of alloxan prevents the diabetogenic action of the latter.[1] This suggests that glutathione may represent one of the natural protections of the body against alloxan or alloxan-like substances.

Griffiths[4] artificially lowered the blood glutathione in 4 rabbits from an average of 38 to 18-23 mg/100 cc by feeding a diet de-

* This work was aided by grants from the American Cyanamid Co., and the Diabetic Fund.

1 Lazarow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 441.

2 Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, 1945, **157**, 525.

3 Bruckmann, G., and Wertheimer, E., *J. Biol. Chem.*, 1947, **168**, 241.

4 Griffiths, M., *J. Biol. Chem.*, 1948, **172**, 823.

TABLE I.
Effect of a Cystine and Methionine Deficient Diet on Weight, Hemoglobin, and Blood Glutathione in Rabbits.

Rabbit No.	Weeks of diet	Wt, g		Hemoglobin, g/100 cc		Blood reduced glutathione, mg/100 cc	
		Initial	Final	Initial	Final	Initial	Final
D1	8	1759	1050	—	5.0	32	20
D2	8	1710	1410	—	5.0	45	22
(2)*	6	1700	1110	5.4	4.7	25	21
D3	11	1775	1222	—	5.1	46	27
D4	7	1952	1493	—	—	56	27
D5	9	1341	1275	—	6.2	46	28
D6	6	1767	1360	—	6.4	44	21
(2)*	7	2180	1280	9.4	7.1	43	17
D7	5	1720	1555	9.3	4.6	50	15
D8	16	1975	1250	11.3	3.8	49	29
D9	12	2480	1810	10.0	5.0	44	33
D10	4	3120	2640	8.8	7.8	44	19
D11	14	3210	1950	11.1	5.3	48	38
D12	12	2980	3015	10.6	7.9	38	19

* After an interval of 1 to 2 weeks on usual rabbit pellet diet.

ficient in cystine and methionine. Uric acid, in dosage of 1 g/kg, was then injected intraperitoneally and the blood sugar determined. He reports that a moderate hyperglycemia ensued by the 2nd day after the injection and remained for 4-5 days. Thereafter normal blood sugar levels were regained.

This paper describes at attempt to repeat Griffiths' experiments and also to determine if decreasing the blood glutathione by bleeding renders the animal sensitive to the production of hyperglycemia when given uric acid.

Methods and Materials. Male albino rabbits weighing 1.3 to 3.2 kg were used and were fasted for 16 to 18 hours before the injection of uric acid.

Griffiths' modification[4] of a cystine and methionine deficient diet described by Haag and Wright[5] was used and this was fed to each animal for 4 to 16 weeks to lower the blood glutathione. An attempt was made to secure blood glutathione levels under 25 mg/100 cc before the injection of uric acid.

In the hemorrhage experiments varying amounts of blood were removed by repeated cardiac punctures. Approximately 30 cc were removed daily or almost daily and bleeding was continued until a low blood glutathione was obtained in 5 rabbits. Uric acid was then injected.

Control rabbits on the usual laboratory diet of Park and Pollard rabbit pellets were also injected with uric acid.

Uric acid (Eastman Kodak or Coleman and Bell Co.) was administered intraperitoneally in dosage of 1 g/kg in a suspension in 20 cc of water. Blood sugar and blood uric acid concentrations were determined before and at intervals after the uric acid injection.

Blood reduced glutathione was determined by the Potter and Franke[6] modification of the Benedict and Gottschall[7] method adapted to the Leitz photoelectric colorimeter. Blood uric acid was determined by the method of Folin.[8] Blood sugar was determined by the Folin and Malmros[9] micromethod and in some cases, to decrease the reducing effect of the uric acid on the reagents, Nelson's[10] blood sugar method was used.

Results. It was found difficult to lower the blood glutathione in rabbits by feeding the

[5] Haag, J. R., and Wright, L. D., *J. Nutrition*, 1940, **19**, 563.

[6] Potter, V., and Franke, K., *J. Nutrition*, 1935, **9**, 1.

[7] Benedict, S. R., and Gottschall, G., *J. Biol. Chem.*, 1932, **99**, 729.

[8] Folin, O., *J. Biol. Chem.*, 1930, **86**, 179.

[9] Folin, O., and Malmros, H. J., *J. Biol. Chem.*, 1929, **83**, 115.

[10] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

TABLE II.
Effect of Hemorrhage on Blood Reduced Glutathione Concentration in Rabbits.

Rabbit No.	Duration of bleeding period (days)	Amt. of blood withdrawn (cc)	Hemoglobin, g/100 cc		Blood glutathione, mg/100 cc	
			Initial	Final	Initial	Final
H1	6	53	8.4	5.9	35	23
H2	3	78	9.8	5.2	38	19
H3	9	109	10.3	8.1	42	42
H4	11	312	9.8	3.8	33	26
H5	3	85	7.8	3.4	33	17
H6	2	122	9.6	4.1	38	20

sulfhydryl-deficient diet as the animals showed anorexia, loss of weight, marked loss of hair, anemia and generalized weakness. Of 21 rabbits on the diet 9 died before the blood glutathione was lowered to 25 mg/100 cc. The effect of the diet on weight, hemoglobin and blood glutathione in the remaining 12 animals is shown in Table I. Only 5 of these rabbits (D1, 2, 6, 7, 10) showed a lowering of blood glutathione to 22 mg or less in 4 to 8 weeks of diet and 1 (D12) in 12 weeks. The remaining 6 were injected with uric acid although the blood glutathione was still 27 to 38 mg/100 cc after 9 to 16 weeks of the diet. Unless the blood glutathione dropped markedly in the first 4 to 8 weeks it seemed that it was unlikely to fall to 25 mg or less with a prolonged diet.

At the suggestion of Griffiths[11] 18 young rabbits weighing 0.5 to 1.0 kg were put on a modified diet containing one-half the protein of the original diet but none of these survived beyond 4 weeks. Six adult rabbits also failed to survive beyond 4 weeks on this reduced protein diet.

The effect of hemorrhage on the blood glutathione is shown in Table II. In 4 rabbits (H1, 2, 5, 6) there was a reduction to 23 mg/100 cc or less with removal of 53 to 122 cc of blood in 2 to 6 days but 2 others (H3,4) were apparently better able to compensate for the blood loss. This compensation has been observed in patients with anemia.[12] One of our rabbits showed no lowering of blood glutathione after 109 cc of blood were removed in 9 days and in another there was only moderate lowering after removal of 312 cc in 11 days.

Table III shows the effect of intraperitoneal injections of uric acid into control rabbits and into those made sulfhydryl-deficient by diet or bleeding. In each group, including the controls, most of the animals had apparent hyperglycemia for several hours after the uric acid injection but by 24 hours the blood sugar was normal. Such initial hyperglycemia did not occur in those animals which failed to show an elevation of the blood uric acid. In 2 animals (D6, C2) a second injection of uric acid at a later date gave the usual increase in blood uric acid accompanied by apparent hyperglycemia. By the Nelson sugar method which is less affected by a high blood uric acid, the hyperglycemia was less but in several cases quite definite.

After the first 24 hours, when the blood uric acid was always negative, one of the rabbits on the deficient diet (D6) showed a marked transitory hyperglycemia reaching 366 mg/100 cc 2 days after injection of uric acid and one other (D4) a mild hyperglycemia of 200 mg on the 5th day. In the hemorrhage group one rabbit (H4) showed a transient hyperglycemia after uric acid reaching 218 mg on the 3rd day. None of the other animals had blood sugar values significantly higher than the control group injected with uric acid.

Rabbit D6 which developed marked hyperglycemia at 2 days was an animal which had failed to show uric acid in the blood after it had been injected intraperitoneally. When D6 was reinjected 8 weeks later the blood

11 Griffiths, M., personal communication.

12 Pickard, R. J., and Marsden, C. S., *J. Lab. and Clin. Med.*, 1933, **19**, 395.

TABLE III.
Effect of Uric Acid Injection on the Blood Sugar in Rabbits.

No.	Group	Blood-glutathione when injected (mg/100 cc)	Highest blood uric acid noted after injection (mg/100 cc)	Blood sugar (mg/100 cc) Time after uric acid: Hours 0	Hours 1	Hours 3	Hours 5	Days 1	Days 2	Days 3	Days 4	Days 5	Days 6
				Fasting values				Non-fasting values‡					
D1	Diet*	20	28	135	160	120	—	48 dead					
D2	”	22	41	128	205	235	214	102	149	147	165	137	137
	(2)	21	28	124	246	260	218	124	181	118	139	130	139
D3	Diet	27	trace	105	90	80	116	80	114	124	120	—	120
D4	”	27	20	114	160	135	—	105	132	142	160	*200*	160
D5	”	28	15	129	180	165	133	95	133	89	116	—	120
D6	”	21	neg.	107	128	135	139	153	*366*	*352*	*278*	*205*	183
	(2)	15	31	130	211	214	233	114	155	139	114 dead		
D7	Diet	15	trace	135	155	149	139	143	149	135	124	—	125
D8	”	29	10	103	260	354	368	84 dead					
D9	”	33	neg.	124	103	112	67	87	103	95	102	—	95
D10	”	19	7	107	143	147	135	116	145	122	127	127	109
D11	”	38	33	149	185	171	130	178	104	127	27 dead		
D12	”	19	7	105	260	294	240	124	128	130	137	143	129
H1	Hemorrhage	23	16	171	211 died								
H2	”	19	13	141	208	250	239	120	153	130	139	130	
H3	”	42	3	143	155	147	128	122	109	109	112	124	
H4	”	26	7	124		174		124		*218*	194	165	165
H5	”	17	neg.	124	135	128		120	135	120	135		120
H6	”	20	trace	116	128	114	112	133	135	130		130	
C1	Control†	57	11	85	200	165	169 dead						
C2	”	38	neg.	132	124	128	92	118	151	156		137	128
	(2)	38	11	124	188	294	294	114	120	120		124	
C3	”	59	14	92	135	137		101	120	114	120		
C4	”	47	8	95	135	149	143	107	116	109	95		
	(2)	42	14	101	151	153	167	101 reinjected daily blood sugars normal					

* Cystine and methionine deficient diet.
† Park and Pollard rabbit pellet diet.
‡ For convenience in interpretation, all blood sugar values of 160 mg or above at 1 day or later, appear in *italic* type.

uric acid rose to 31 mg/100 cc but no hyperglycemia ensued after 24 hours. Rabbit D4 with a mild hyperglycemia reaching 200 mg did show a blood uric acid elevation to 20 mg/100 cc after injection.

In one rabbit with blood glutathione lowered to 25 mg/100 cc, neutralized sodium urate in 1.2% solution was injected intravenously. Five injections of 0.4 mg/kg each were given in 15 days. The blood sugar remained normal throughout.

Discussion. The cystine and methionine deficient diet theoretically lowers the blood glutathione, a tripeptide containing glycine, glutamic acid and cysteine, by depriving the body of cystine. The rabbits in our experiment did not tolerate this diet well and it was therefore difficult to secure rabbits with lowered blood glutathione for uric acid injection.

No explanation is apparent why one animal showed marked hyperglycemia for 4 to 5 days after uric acid while others which had blood glutathione values as low or lower failed to do so. Only 2 of the 8 rabbits with glutathione of 27 mg or less showed hyperglycemia.

We cannot explain why only a trace or no blood uric acid could be detected after its intraperitoneal injection in 7 instances whereas others attained blood uric acid levels up to 41 mg/100 cc at the same time intervals, nor can we explain the different response in the same animal with a second injection.

A rough correlation existed in most cases between the apparent hyperglycemia observed in the first few hours after injection and the height of the blood uric acid, which may be explained in part by the fact that uric acid reduces the ferricyanide blood sugar reagent. However there were several instances of rising blood sugar with falling uric acid levels and also some high blood sugar values by the Nelson method.

Since all animals became quite anemic on the cystine and methionine deficient diet, and since almost all of the glutathione in normal blood is in the red cell, attempts were made to lower the glutathione by repeated bleedings. In the small group of animals with the blood glutathione lowered in this manner transient hyperglycemia was observed at 3 to 4 days in 1 of 4 rabbits.

Although the results of these experiments were variable and in most cases negative it was thought worthwhile to report them in view of the current interest in glutathione in the prevention of diabetes and the possible role of uric acid or related compounds in the production of diabetes.

In spite of the large proportion of negative results the marked hyperglycemia in one rabbit and the moderate hyperglycemia in 2 others given uric acid with a lowered blood glutathione suggest that further investigation of this subject may be worthwhile. However the impression is gained that if uric acid under these conditions is diabetogenic, its action is very weak.

Summary. Attempts were made to confirm the report of Griffiths that transient diabetes follows the intraperitoneal injection of uric acid into rabbits in which the blood glutathione has been lowered by feeding a diet deficient in cystine and methionine.

In our experiment 21 rabbits were fed the deficient diet. Twelve animals surviving the dietary restriction were injected with uric acid although only 6 had a blood glutathione below 23 mg/100 cc. One of these had a definite transient hyperglycemia for 5 to 6 days after the uric acid injection. No blood sugar changes were seen in the other animals after 24 hours. One rabbit with a blood glutathione of 27 mg/100 cc showed a moderate transient hyperglycemia.

When the blood glutathione was lowered by bleeding instead of diet only one of 4 rabbits injected with uric acid showed a transient hyperglycemia after 24 hours.

It is concluded that if uric acid is diabetogenic under these conditions its action is weak.

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17265. Effect of Germination on Phytin Content and Phytase Activity of Some Common Indian Pulses.

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Phosphorus present in foodstuffs as phytin is largely unavailable for nutrition. Studies have been made by several workers of the availability of phytin phosphorus. Krieger, Bunkfeldt and Steenbock[1] observed that when rats were fed a cereal ration of normal calcium content the utilization of phosphorus from phytin was markedly enhanced by the addition of vitamin D. Boutwell, Geyer, Halverson and Hart[2] studied the availability of wheat bran phosphorus, which contains about 85% of phosphorus as phytin, in rats and observed that an adequate intake of vitamin D increased the utilization of phytin phosphorus as measured by bone ash. Pringle and Moran[3] and Widdowson[4] reported that phytin in wheat meal or white flour is partly destroyed during the process of preparation of bread. Pulses are rich in phytin. The vitamin content of pulses is found to increase during the process of germination.[5,6] It was, therefore, considered desirable to investigate whether the phytin content of pulses is diminished during germination.

Methods. One gram of clean and dry seeds of pulses were placed in a washed and sterilized petri dish. The seeds in each dish were soaked daily with 1 cc of redistilled water for a period of 5 days. Phytin phosphorus content of the seeds was estimated both before and during the course of germination up to 5 days. Total phosphorus content of the seeds was also estimated.

Total phosphorus. The seeds were finely ground and digested in a Kjeldahl flask with 10 cc of concentrated sulfuric acid and 1 cc of 60% perchloric acid for half an hour till the digestion was complete and the contents were diluted to a definite volume and the total phosphorus content was estimated by the method of Fiske and Subbarow.[7]

Phytin phosphorus. 1 g of the material was extracted with 50 cc of N/2 hydrochloric acid in a glass stoppered bottle for 2 hours and filtered. The phytin present in the filtrate was precipitated as the insoluble iron salt. The insoluble salt was converted to soluble sodium salt which was digested with sulfuric acid and perchloric acid mixture and the inorganic phosphate thus obtained was estimated by the method of Fiske and Subbarow.[7]

Phytase activity. 5 g of pulses, before or during the course of germination, were ground and extracted for 12 hours with 50 cc of water saturated with toluene. The extract was centrifuged. Inorganic phosphorus in a 10 cc portion of the centrifugate, representing 1 g of the pulse, was estimated and to another 10 cc of the extract was added acetate buffer of pH 5.2 and a substrate containing

TABLE I.
Mg of Phytin Phosphorus Present in 100 g of Pulses.

Pulses	Days of germination					
	0	1	2	3	4	5
Phaseolus mungo	156	156	101	96	95	95
Phaseolus radiatus	158	138	117	110	109	109
Cicer arietinum	138	125	117	97	80	80
Dolichos lablab	138	127	114	104	105	105

[1] Krieger, C. H., Bunkfeldt, R., and Steenbock, H., *J. Nutrition,* 1940, **20**, 7.

[2] Boutwell, R. K., Geyer, A. P., Halverson, A. W., and Hart, E. B., *J. Nutrition,* 1946, **31**, 193.

[3] Pringle, W. J. S., and Moran, T., *J. Soc. Chem. Indust.,* 1942, **61**, 108.

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[6] French, C. E., Berryman, G. H., Goorley, J. T., Harper, H. A., Harkness, D. M., and Thacker, E. J., *J. Nutrition,* 1944, **28**, 63.

[7] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.,* 1925, **66**, 375.

TABLE II

γ of Phosphorus Liberated from the Substrate Containing 10 mg of Phosphorus in the Form of Sodium Phytate by the Phytase Present in 1 g of the Pulse Either Before or After Germination.

	Days of germination					
Pulses	0	1	2	3	4	5
Phaseolus mungo	39	46	66	82	91	91
Phaseolus radiatus	167	179	200	206	208	208
Cicer arietinum	39	43	47	50	50	50
Dolichos lablab	172	186	191	192	191	192

10 mg of phosphorus as sodium salt of phytin. The mixture was incubated at 35°C for 6 hours. The phosphorus liberated from phytin was estimated by the method of Fiske and Subbarow.[7]

Results. The total phosphorus content in mg per 100 g of *phaseolus mungo, phaseolus radiatus, cicer arietinum* and *dolichos lablab* was respectively 375, 302, 219 and 247. Phytin phosphorus values and phytase activities of the pulses are given in Tables I and II.

Discussion. Phytin phosphorus values of all the 4 varieties of pulses studied were found to decrease gradually during the process of germination and the maximum lowering of the value occurred either on the third or on the fourth day of germination. The phytase activity of the pulses was found to increase during the course of germination and when the phytin content was minimum the phytase activity was maximum. This suggests that the enzyme phytase which is formed during the process of germination acts upon the phytin phosphorus and partly destroys it. It has been observed by French *et al.*[6] that 48 hours after germination of peas there is a considerable increase in the inositol value of the pulse. Phytin being the calcium or magnesium salt of inositol hexaphosphoric acid, the increased inositol value of the pulse after germination might be due to the breaking down of phytin by the enzyme phytase. The germinated pulse is therefore nutritionally superior to the ungerminated one.

Summary. Phytin content and phytase activity of 4 pulses have been estimated both before and for varying periods after germination. Phytin content gradually diminished along with the increase in the phytase activity of the germinating pulse which was maximum either on the third or on the fourth day of germination.

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17266. Creatinine, Potassium, and Virus Content of the Muscles Following Infection with the "Coxsackie Virus."*

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A number of specimens of feces from children exhibiting symptoms of poliomyelitis have yielded viruses that produce degeneration of the skeletal muscles of suckling mice and hamsters.[1,2] The anatomic response in man is not known but the clinical records of patients from whose feces virus was isolated suggest that muscle weakness or paralysis is common and sometimes persists for months. While considering means of testing patients for muscle degeneration, it occurred to us that creatine losses probably accompany the destruction of the striated muscle cells and,

* This agent is being called "Coxsackie Virus" since the first recognized human cases were residents of that New York village.

1 Dalldorf, Gilbert, and Sickles, G. M., *Science*, 1948, **108**, 61.

2 Dalldorf, Gilbert, Sickles, G. M., Plager, Hildegard, and Gifford, Rebecca, *J. Exp. Med.*, 1949, **89**, 567.

TABLE I.
Concentration of Total Creatinine in Urine of Normal and Infected Suckling Mice.

		Total creatinine					
		Normal animals		Infected animals			
Group	Age (days)	No. in group	mg/ml	No. in group	Days following inoculation	Paralysis	mg/ml
1	6	22	0.45	22	1	—	0.3
	7	20	0.50	24	2	+	1.5
	8	20	0.50	18	3	++	2.7
	9	10	0.60	3	4	++++	2.0
2	6	22	0.30	23	1	—	0.33
	7	22	0.25	23	2	—	1.01
	7½	22	0.47	22	2½	+	3.00
	8	19	0.40	21	3	++	3.00
	8½	18	0.25	14	3½	+++	2.72
	9	17	0.26	8	4	++++	2.38
3	7	16	0.22	18	1	—	0.81
	7½	16	0.29	17	1½	—	1.05
	8	16	0.05	18	2	+	1.92
	8½	16	0.45	18	2½	++	3.50
	9	16	0.30	18	3	+++	3.50

further, that, since the muscle weakness in some patients was migratory and transient, serum potassium concentration should be tested. It has been impossible to secure suitable serum specimens from immature mice but the potassium and total creatinine values of the muscles have been determined, as well as urinary creatinine. The present report of these results includes, in addition, certain observations on the infectivity of the muscles.

Methods. The muscle samples for chemical analyses taken from immature mice at various times following intraperitoneal injection of brain suspensions of the T. T. strain, were placed in tared and sealed containers and weighed within 2 hours. The urine samples were obtained by lightly pressing on the lower abdomen with a wooden applicator. Since suckling mice do not urinate without external stimulus, small urine specimens were readily procured. In order to avoid possible differences between litters, the mice were first pooled and redistributed at random[3] before inoculation.

Potassium was determined by the phosphotungstate method of Van Slyke and Rieben.[4] The samples for muscle creatinine determinations were prepared according to the directions of Rose, Helmer, and Chanutin,[5] and creatinine was determined by the colorimetric method of Folin and Wu.[6]

Excised muscle fragments were initially used for infectivity tests. Subsequently, preparations consisting of pools of the extremities, including bone and fascia, were used.

Potassium and Creatinine Determinations. Potassium determinations were made of samples of muscle from 24 normal and 23 paralyzed suckling mice. The mean for the first group was 2.96 ± 0.16 mg per g of tissue, and that of the experimental group was 2.08 ± 0.28 mg per g. The number following the ± sign is the mean deviation. The greater value for the experimental group may be due to the uneven distribution of the muscle lesions. A striking feature of this experience was that all but one of the potassium values of the paralyzed animals were less than the least value of the controls.

Creatinine determinations were made of 8 samples of each of the above groups. The

[3] Thompson, W. R., *Bact. Rev.*, 1947, **11**, 115.

[4] Van Slyke, D. D., and Rieben, W. K., *J. Biol. Chem.*, 1944, **156**, 743.

[5] Rose, W. C., Helmer, O. M., and Chanutin, Alfred, *J. Biol. Chem.*, 1927, **75**, 543.

[6] Folin, Otto, and Wu, Hsien, *J. Biol. Chem.*, 1919, **38**, 81.

TABLE II.
Infectivity of Pools of Legs and of Brains of Paralyzed Mice.

Source of virus	Test animals	Dilutions of virus								
		10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9
Suckling mice legs	Suckling mice 4-5 days				8/8	8/8	8/8	7/8	0/7	1/7
" " "	Weanling mice 7-8 g	9/9	8/10	3/10	1/10					
" " "	" " 8-10 g	0/10	0/10	0/10	0/10					
" " "	Mice 10-12 g	0/5								
" " "	" 14 g	0/5								
Suckling mice brains	Suckling mice 4-5 days	8/8	8/8	4/8	0/7	0/7				
" " "	Weanling mice 7-8 g	1/10	1/10	1/10	0/10	1/10				
Weanling mice legs	Suckling mice 7 days				7/7	8/8	7/7	4/8		
" " "	Weanling mice 7-8 g	7/7	4/7	2/7	2/7					
Weanling mice brains	Suckling mice 7 days	7/7	8/8	2/7	0/7					
" " "	Weanling mice 7-8 g	0/7	0/7	0/7	0/7					

Note: No. of mice paralyzed or dead/No. of mice inoculated.

mean concentration of total creatine in the control group was 1.95 ± 0.15 mg per g and for the experimental group, 0.96 ± 0.23 mg per g.

The concentration of total creatinine (creatine plus creatinine) in the urine of various normal and infected suckling mice is shown in Table I. The mean for the normal animals was 0.35 mg per ml, while the concentration in diseased animals was frequently ten times as great. Increased excretion of creatinine occurred in two instances before the onset of symptoms. It should be noted that the samples from the sick mice were smaller. This is thought to have been due to the smaller size of the affected animals, since a comparison of their urinary pigments did not indicate that their urine was more concentrated. Stunting is characteristic of suckling mice infected with the virus. Whether this is a result of intrinsic changes or inferior nursing we do not know.

Infectivity of muscle. The muscles of paralyzed mice are more infectious than the tissues of the central nervous system. Suspensions prepared from pooled legs are roughly ten thousand times as infectious for suckling mice as those prepared from brains (TableII). When 10- to 12-g mice are inoculated with brain suspensions the organs contain little or no virus and while an occasional focus of muscle degeneration may sometimes be found, the mice exhibit no recognizable signs of infection. On the other hand, 10- to 12-g mice inoculated with muscle suspensions are sometimes paralyzed and weanling mice (7 to 8 g) are usually paralyzed. These observations prompted us to attempt to adapt the virus to 10- to 12-g mice by alternate passage[7-10] through suckling mice. Six alternate passages have shown no increase in infectivity and the brains and muscles of the older mice remain less infectious than those of suckling animals. Serial passages in older mice have also failed.

The greater infectivity of muscle suspensions provided effective antisera from recovered hamsters and a rhesus monkey. The monkey showed no signs of infection following intramuscular inoculation but developed humoral antibodies after a series of intraperitoneal injections. Paralysis and muscle lesions were induced in one of 6 new-born guinea pigs inoculated intraperitoneally with 0.5 ml of a 10% suspension of hamster legs. The paralyzed extremity was infective for suckling mice, while the brain was not.

Virus has been found in the feces of 7- to 8-g mice on the day of paralysis but not in the feces of 18-day-old hamsters that had been paralyzed for 2 days.

Limited tests with a few of the strains of

[7] Baker, J. A., *Amer. J. Vet. Res.*, 1946, **7**, 179.

[8] Baker, J. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 183.

[9] Koprowski, Hilary, James, T. R., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 178.

[10] Dean, D. J., and Dalldorf, Gilbert, *J. Exp. Med.*, 1948, **88**, 645.

virus that affect both central nervous system and muscle have shown that suspensions of legs are either less virulent or about as virulent as suspensions of brains. This conforms with the signs of infection, with the histologic findings, and with the degree of creatinuria associated with the two types of disease.

Discussion. Whether or not these observations have application in clinical medicine is unknown. Creatinuria follows paralytic poliomyelitis[11-13] but has been assumed to be a sequel of neurone destruction. Since there is some evidence that muscle degeneration may occur in the initial stages of the disease,[14] the subject should be re-examined. Hassin described a case of poliomyelitis of the Landry type in which the lesions were restricted to the muscles.[15] Clawson is reported to have found no muscle lesions in a series of 22 cases of acute poliomyelitis[16] but Dr. Kornel Terplan, Buffalo General Hospital Laboratory, provided us with histologic preparations from a case of fatal bulbar poliomyelitis in which extensive muscle lesions similar to those which occur in suckling mice were seen.

The infectivity of the muscles is consistent with the morphologic changes and reaffirms the peripheral nature of the paralysis in the mice. It is interesting that the higher titer of the muscle preparations makes it possible to induce paralysis in mice of an age-group that is resistant to infection with brain suspension. While these findings modify, they also confirm the original observation of the remarkable natural immunity of mice and hamsters following weaning. This is further supported by failure to adapt the T.T. strain to weaned mice by alternate passage through susceptible animals.

Summary. Infection of unweaned mice with certain strains of "Coxsackie virus" is followed by loss of muscle potassium and creatinine and by creatinuria.

The muscles of paralyzed mice are highly infectious.

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16 Bell, E. T., The Progressive Pathology of Poliomyelitis. In: Poliomyelitis. Papers and Discussions presented at the First International Poliomyelitis Conference. J. B. Lippincott, Philadelphia, 1949, p. 135.

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17267. The Role of Complement in the Lysis of Leucocytes by Tuberculoprotein.*

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(Introduced by J. H. Mueller.)

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The nature of the mechanism underlying delayed or tuberculin-type hypersensitivity has long interested students of immunology. Whereas many workers have attempted to demonstrate an antibody in tuberculin allergy which was similar in properties and mode of action to antibodies known to be responsible for anaphylactic or Arthus type of hypersensitivity, the preponderance of such studies has led to the assumption that cells, rather than serum antibodies, were the mediators of tuberculin hypersensitivity. Holst[1] first noted

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‡ Stanley W. Tausend Fellow in Medicine.

1 Holst, P. M., *Tubercle*, 1922, **3**, 337.

decreased phagocytic activity and loss of differentiation between the nucleus and cytoplasm when tuberculin was added to leucocytes derived from tuberculous animals but no deviation from normal when horse serum was added to leucocytes from guinea pigs sensitized to horse serum. Similarly Stewart *et al.*[2] observed that tuberculin killed leucocytes derived from the tuberculous guinea pig while the leucocytes of a guinea pig sensitized to crystalline egg white were not affected by the addition of that specific antigen.[3] These and other studies[4-9] have distinguished this cytotoxic role of antigen on cells of tuberculin-type allergic animals from the harmless response of cells to antigen in the anaphylactic forms of hypersensitivity. In the latter instance, serum antibody is felt to be the crucial factor in the hypersensitivity manifestations. The failure to transfer passively tuberculin sensitivity by means of the serum of a sensitized animal and the successful transfer of such sensitivity with cells of the tuberculous guinea pig by Chase[10] and others[11-13] have given further support to the concept that cells, rather than serum antibody, were the prime mediators of tuberculin type hypersensitivity.

The studies of Favour,[14] using a one-hour period of *in vitro* observation, have provided a simpler method of determining the cytotoxicity of tuberculin and subsequent investigation in this laboratory has aimed at elucidating the mechanism of tuberculin allergy by this *in vitro* technic. Earlier work[15] with white cells of tuberculous humans indicated that tuberculin was specifically toxic to these cells. It was further assumed that complement was essential for such cytolysis since no tuberculin cytolysis was observed if the diluting serum was heated at 56°C for 30 minutes.

A more recent report from this laboratory,[16] however, has shown that it is unnecessary to use "sensitized tuberculous cells"§ to demonstrate short term tuberculin cytolysis. *In vitro* lysis of leucocytes by tuberculo-protein has been found to be dependent on the presence of tuberculous plasma (or serum). Further studies[17] now make it apparent that the active component of tuberculous plasma is a heat labile globulin. This finding of an antibody-like factor in tuberculous plasma (or serum) which is responsible for the cytotoxic action of tuberculin on tuberculous as well as on normal leucocytes‖ prompted a reinvestigation of the role of complement in this reaction. Because of the heat lability of the plasma factor, it was necessary to resort to other methods of removing complement completely from tuberculous plasma (or serum).

Experimental. As in previous experiments,[16-17]§ white blood cells from a normal, healthy tuberculin-negative subject were separated, washed 3 times in saline and used as the target cell for determining tuberculin

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13 Stavitsky, A. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 225.

14 Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 269.

15 Fremont-Smith, P., and Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 502.

16 Miller, J. M., Favour, C. B., Wilson, B. A., and Umbarger, M. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 738.

§ White cells from a patient having active tuberculosis.

17 Miller, J. M., Favour, C. B., Wilson, B. A., and Umbarger, M. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 287.

‖ Cells from a normal, healthy, tuberculin-negative subject.

cytolysis, thus leaving the tuberculin antigen, the tuberculous plasma factor and complement as the 3 vital determinants of eventual cell breakdown. Plasma (or serum) was obtained from patients hospitalized for active pulmonary tuberculosis whose plasma had previously been shown to contain the active plasma factor. Plasma from normal, healthy tuberculin-negative donors was used as a control. In order to remove complement completely from this plasma without inactivating the heat-labile plasma factor essential for cell lysis, two separate antigen-antibody systems were used. In the first instance, pneumococcal type III polysaccharide and antipneumococcal type III rabbit antiserum (previously found to produce a precipitate with antigen diluted 1:2,000,000)¶ were added to tuberculous plasma and normal plasma in such dilution as to give a precipitate in the zone of equivalence for this antigen-antibody system and thus fix complement. This mixture was incubated at 37°C in a water bath for 2 hours and then in the ice box at 10°C for 2 days. As a second complement-fixing antigen-antibody system, crystalline bovine albumin and anti-bovine albumin rabbit serum (previously found to produce a precipitate with antigen diluted 1:800,000)¶ was similarly added to tuberculous and normal plasma so as to yield precipitate in the zone of equivalence for that system. Complement titers were determined on the untreated tuberculous and normal plasma as well as on such plasma to which had been added the pneumococcal polysaccharide and bovine albumin systems in order to confirm the complete absence of complement from the latter two systems.

In estimating the amount of complement activity present in individual untreated sera, a modification of the method proposed by Mayer *et al.*[18] was employed. Using 0.5 ml amounts of 4% sheep cell suspensions, sensitized with four units of high titer amboceptor, as the indicator system, the amount of serum necessary to produce 50% hemolysis was determined. The final titer was calculated from the equation

$$C = \frac{D}{V} \times 2.5$$

where C = the number of 50% hemolytic units of complement present per ml of undiluted serum, D = the reciprocal of the dilution of serum used, V = the volume of this dilution necessary for 50% hemolysis, and 2.5 is a factor used in this laboratory to make the results referable to those of our determinations which use a system employing only 0.2 ml of sensitized sheep cells. By this method normal human sera usually have complement titers in the vicinity of 300 units per ml. Plasma may show slightly lower values because of the anticoagulant used. All dilutions were made with a veronal buffered saline containing optimal quantities of calcium and magnesium ions.[19]

In those sera from which complement had been removed, a titration procedure designed to detect complement sufficient to produce 50% hemolysis was obviously not applicable. It was necessary to detect minimal hemolysis. Accordingly, to 0.2 ml suspensions of sensitized sheep cells were added the test sera in dilutions just sufficient to abolish non-specific hemolysis and/or anticomplementary factors. The final volume was 0.8 ml. After incubation for 40 minutes at 37°C the cells were centrifuged and the supernatant examined for evidence of hemolysis. In no instance, with either of the complement-fixing antigen-antibody systems used, was there any demonstrable complement activity remaining in the tuberculous or normal sera.

In order to demonstrate that the cytolysis promoting capacity of such plasma could be restored by the readdition of complement, freshly drawn type AB serum from a tuberculin-negative subject was titered for complement and added to the "decomplemented" plasma in separate tubes. As the tuberculin antigen in all experiments, Old Tuberculin,

¶ Kindly supplied by M. H. Kaplan of the Department of Bacteriology, Harvard Medical School.

[18] Mayer, M. M., Eaton, B. B., and Heidelberger, M., *J. Immunol.*, 1946, **53**, 31.

[19] Mayer, M. M., Oster, A. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84**, 535.

TABLE I.

Effect of Complement on *in vitro* Lysis of Normal Leucocytes by Tuberculin in Presence of Normal and Tuberculous Plasma.

	Complement 50% hemolytic units per ml																
Normal cells		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
Tuberculous plasma	217	0.3	0.3	0.3	0.3												
Tuberculous plasma "decomplemented"*	0					0.6	0.6	0.6	0.6								
Normal plasma	234									0.3	0.3	0.3	0.3				
Normal plasma "decomplemented"*	0													0.6	0.6	0.6	0.6
Fresh type AB serum	214			0.1	0.1			0.1	0.1			0.1	0.1			0.1	0.1
Tuberculin antigen		0.1		0.1		0.1		0.1		0.1		0.1		0.1		0.1	
Saline		0.1	0.2		0.1	0.1	0.2		0.1	0.1	0.2		0.1	0.1	0.2		0.1
Total WBC																	
5 min.		9,650	9,330	9,770	9,330	6,270	6,330	6,130	6,260	10,110	10,190	7,725	7,320	5,400	5,480	4,980	6,120
60 min.		7,670	9,260	7,790	9,200	6,300	6,310	4,930	6,330	10,060	10,280	7,650	7,270	5,330	5,470	5,000	6,080
% Decrement		—20.5	—0.7	—20.2	—1.4	+0.5	—0.5	—19.5	+0.7	—0.5	+0.7	—0.8	—0.7	—1.2	—0.5	+0.2	—0.5

* To remove complement from 2 ml of plasma, 1 ml of a 1:1000 solution of crystalline bovine albumin and 1 ml of anti-bovine albumin rabbit serum was added; thus, final dilution of plasma was 1:2.

prepared as described earlier,[20] was used. From previous experiments, it was found best to use cells and plasma from homologous blood groups.

The methods of preparing and washing white cell concentrates, diluting these cells in the various plasma (or sera) and cell counting to estimate cytolysis were the same as described previously.[16,21]

Results. The results of a typical experiment are recorded in Table I. In this instance, complement was removed by means of the crystalline bovine albumin system but identical findings were observed with the pneumococcal polysaccharide antigen-antibody system.

1. As reported earlier,[16] a portion of thoroughly washed white blood cells from a healthy, tuberculin negative subject undergoes significant cytolysis when incubated for one hour at 37°C with freshly drawn tuberculous plasma and tuberculin antigen. No such cytolysis occurs if the plasma of a tuberculous patient is replaced by the plasma of a tuberculin-negative subject.

2. Removal of all complement from tuberculous plasma by means of an unrelated complement-fixing antigen-antibody system results in the loss of the ability of such plasma to affect cytolysis of white blood cells. The successful removal of all complement by this technic has been confirmed by the sheep cell hemolysis method described above. It can be noted that 0.6 ml of "decomplemented" plasma was used instead of the 0.3 ml used for fresh plasma. This was done since the complement fixing procedure resulted in a 1:2 dilution of the original plasma.

3. The addition to the system of active complement in the form of freshly drawn type AB tuberculin-negative serum restored completely the capacity of "decomplemented" tuberculous plasma to cause white cell destruction in the presence of Old Tuberculin.

4. Plasma from a tuberculin negative subject continued to have no effect on white blood cells even after the addition of fresh complement.

5. The addition of further complement of known titer to this system in the form of freshly drawn AB serum from a tuberculin-negative donor does not further enhance the tuberculin cytolysis of this system. It may be assumed, therefore, that the tuberculous plasma already has adequate amounts of complement.

Discussion. The present report further elucidates the mechanism of the *in vitro* cytolysis of human white blood cells by tuberculoprotein. Contrary to the original hypothesis in this laboratory that tuberculin cytolysis was a direct toxic effect of tuberculin on the tuberculous "sensitized" cell, a more recent report[16] has shown that even normal white cells of a tuberculin negative donor can be lysed by tuberculin provided a factor in tuberculous plasma is also present. Various properties[17] of this factor suggest that it is a globulin. This work further characterizes such *in vitro* cytolysis as a manifestation of an antigen-antibody inter-action. It now appears evident that complement too is an essential component of this system.

The question of most significance in these studies is whether or not such *in vitro* short term tuberculin cytolysis, dependent as it is on antigen, antibody and complement, may be taken to be a valid analogue of *in vivo* cutaneous tuberculin hypersensitivity. If this be so, then the dependence of the antigen-antibody reaction upon complement has significance in indicating the role of the lytic action of this substance in this type of hypersensitivity. The successful passive transfer of tuberculin hypersensitivity in guinea pigs by Chase,[10] using living cells, allows the possibility that the hypersensitivity developed in the recipient animal may be due primarily to elaboration by the cells of antibody which is identical in properties to the plasma factor demonstrated in this laboratory.

Summary. The essential role of complement in the *in vitro* cytolysis of human white blood cells by tuberculin has been demonstrated, using two different systems of complement removal. In this way, the mechanism

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[21] Favour, C. B., Fremont-Smith, P., and Miller, J. M., *Am. Rev. Tub.*, 1949, **60**, 212.

of *in vitro* tuberculin hypersensitivity has been further elucidated.

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17268. Alloxan Subdiabetes in Rabbits Detected by Modification of Glucose Tolerance by Adrenal Cortex Extract.

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The subdiabetic state may be defined as a phase of pancreatic diabetes characterized by subtle metabolic disorders; these disorders, in contrast to overt diabetes, are unaccompanied by hyperglycemia or glycosuria, and, in contrast to latent diabetes, are not accompanied by impaired glucose tolerance as determined by conventional methods. The existence of such a state, which might or might not be a prediabetic state, has been suspected by many students of human diabetes mellitus.

Although latent diabetes has been demonstrated in various animals,[1-3] subdiabetes and prediabetes have only been demonstrated by retrospective analysis of the records of mothers of overweight babies[4] and of the records of partially pancreatectomized animals.[5,6] The present studies were undertaken in an attempt to produce experimental subdiabetes, and, by exploiting the adrenal cortex-insulin antagonism,[7] to devise a method for its detection.

Methods and Materials. Mongrel and white rabbits weighing 2.5-3.6 kg were used. Their diet consisted of oats, hay, carrots, cabbage, lettuce, and water *ad lib.* The animals were starved for 18-20 hours prior to each experimental procedure, including the alloxan injections. Sugar was determined on ear vein blood by the method of Hagedorn and Jensen;[8] no anticoagulant was used, and the precipitated samples were all permitted to stand, without fluoride, until the end of the third hour, when the determinations were made. Urinary sugar was not followed.

Glucose tolerance was tested by the intravenous injection, during about one minute, of 3 g of glucose in 50% solution, regardless of weight; this was followed by 2-3 cc of physiologic saline. Fasting, 1, 2, and 3 hour blood samples were drawn. The effect of adrenal cortical hormones on the glucose tolerance was studied by injecting aqueous adrenal cortex extract (Upjohn) intramuscularly, ½ cc/100 g body weight, one half hour before the glucose injection; the entire procedure will be called the *A. C. Tolerance Test.* It should be noted that the fasting blood sugar specimens in all A. C. tolerance tests were drawn one half hour after the injection of adrenal cortex extract (A.C.).

One day or more after the completion of preliminary tolerance tests, alloxan monohydrate (Eastman) was rapidly injected into the ear veins of fasted rabbits in 2.5% solution; further tolerance tests were performed, and subsequent doses of alloxan given, if indi-

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TABLE I.

Animal and wt	Date	Alloxan, mg/kg	Glucose tolerance. Blood glucose level in mg/100 cc Date	0	1	2	3	Adrenal cortex-glucose tolerance. Blood glucose level in mg/100 cc Hour Date	0	1	2	3
No. 1			9/9	99	177	101	93	9/23	139	138	115	106
2.9 kg		None										
No. 2			8/26*	111	276	188	—					
2.9 kg	8/30	23										
	10/22	29.3	10/28	101	110	97	95	11/4	77	159	215	206
			11/11	97	111	106	97					
No. 3			9/23	108	104	125	97					
3.0 kg	9/24	22	9/28	113	—	102	117	9/30	92	166	141	127
No. 4			10/7	86	199	139	146					
2.6 kg	10/8	24.2										
	10/15	15	10/26	122	120	110	99	10/28	104	177	139	124
No. 5			10/7	104	154	124	115	10/14	72	173	120	104
2.5 kg	10/15	15										
	10/28	38	11/11	84	208	117	79	11/15	75	213	159	104†
			11/18	113	179	120	88†					
No. 6			11/18	106	154	99	136	11/25	90	238	102	106
3.6 kg	12/7	41	12/16	102	127	101	93	12/19	119	177	145	117
			12/23	102	111	95	90					
No. 7			11/25	95	146	101	65	12/2	—	184	148	131
3.0 kg	12/7	25										
	12/17	20	12/30	101	150	102	99	1/4	113	132	154	127
			1/18	95	150	102	119					
No. 8			9/28	97	170	186	132	9/30	127	241	226	204
2.9 kg		None										

* 7.4 g of 10% glucose injected intraperitoneally.
† Blood specimens drawn 15 minutes after the indicated hour.

cated. The initial and subsequent alloxan doses listed in Table I are approximate since in some instances there were small or moderate amounts of leakage at the hub of the needle, or of subcutaneous infiltration.

Autopsy was performed on rabbit No. 5 within 15 minutes of its death, and on rabbits Nos. 4 and 7 after approximately 8 hours. Pancreas, aorta and kidneys were studied, fixed in formalin, and stained by conventional histologic methods.

Results. Eight rabbits were studied, of which 6 received alloxan on one or two occasions. Blood sugar observations on these animals, before and after alloxan treatment, are presented in Table I. The data in Table I were treated statistically. Five means were computed for each hour of the glucose tolerance tests, using the following series: (1) The initial glucose tolerance tests; (2) the initial A.C. tolerance tests; (3) the glucose tolerance tests following the first effective dose of alloxan; (4) the A.C. tolerance tests immediately following the preceding group; and (5) the ordinary glucose tolerance tests following shortly after the fourth group. Calculations of the standard errors of the differences between each of these 5 blood sugar means were made for each hour (0,1,2, and 3) with the usual equation for small series, and p was determined, in each instance, from Fisher's t table. Where two successive tolerance tests of the same type were performed the later figures were used in computing the means, and only these figures appear in Table I; for the initial glucose tol-

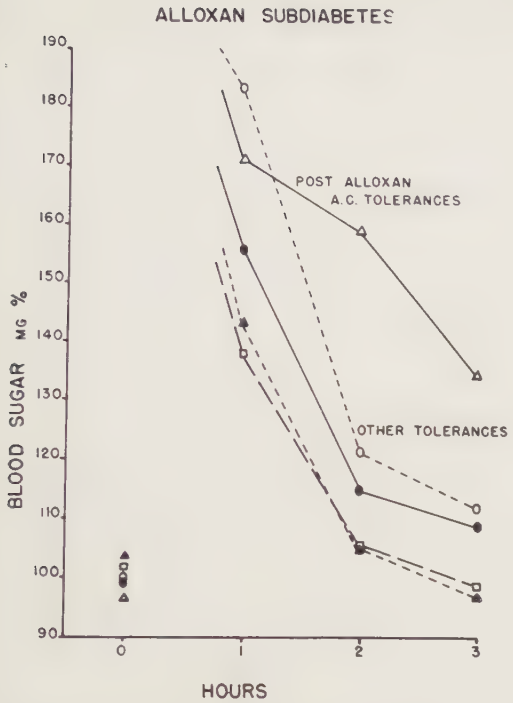

FIG. 1.
Glucose tolerance curves before and after alloxan.
●——● Initial glucose tolerances
○ - - - ○ Pre-alloxan A.C. tolerances
▲ - - - ▲ Post-alloxan glucose tolerances
△——△ Post-alloxan A.C. tolerances
□ – – □ Final glucose tolerances
Each point represents a mean blood sugar value as described in text.

erance tests the earliest figures were used. The control values for Rabbit 2 were not used because of the difference in method (see Table I), and none of the values for Rabbit 8 were used because of its spontaneous diabetes.

Two hours after the injection of glucose the mean blood sugar of the post-alloxan A.C. tolerance tests is 158.8 mg%, this is significantly higher than any of the other blood sugar means at that hour ($p < 0.01$ for all the differences except that between the pre- and post-alloxan A. C. tolerance means, where $p = 0.05$). At three hours the post-alloxan A.C. tolerance mean is 37.2 mg % higher than that of the post-alloxan glucose tolerances; this difference is of moderate significance ($p < 0.05$). No other p values of 0.05 or less were found. Glucose tolerance curves for the five experimental situations have been plotted in Fig. 1, using the same blood sugar means as in the statistical treatment.

Of the 6 rabbits which received alloxan, 4 were retested after a month or more, while one animal (No. 3) died 48 hours after a third dose of alloxan of 25 mg/k, and another (No. 7) died on 5/5 following a month of polydipsia and marked polyuria. Data on the retested animals is presented in Table II. Animal No. 4 subsequently died (4/26) following about 8 weeks of polydipsia and polyuria; it had not been retested since 1/13. Rabbit 5, which developed a diabetic ordinary glucose tolerance, was found to have hind limb paralysis shortly after its last test. Diarrhea supervened and the animal died immediately following the intraperitoneal injection of 60 cc of physiologic saline on the fifth day after this last tolerance test. At autopsy the bladder was found to be distended by about 100 cc of slightly turbid, albumin-free urine containing 179 mg% of sugar. The abdominal aorta exhibited two 0.5 cm long fusiform swellings; the intima in these areas was rough, friable, and pearly with loss of elasticity of the wall. The other organs appeared grossly normal. Histologic sections of the aorta showed medial sclerosis and calcification without significant intimal involvement. The aortas of Rabbits 4 and 7 appeared grossly normal.

Discussion. Intravenous Glucose Tolerance. In order to stabilize test conditions the rabbits were fasted[9] after a period of adequate carbohydrate feeding.[10] A uniform intravenous dose of 3 g of glucose (0.9-1.2 g/kg) was selected for the experiments in order to circumvent variability both in the time and in the degree of absorption of the sugar. Others have found intravenous tolerance methods satisfactory in normal rabbits,[2,11-13] and modification of moderate glucose dosage in

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[10] Martin, E., Haworth, W. N., and Fantus, B., Dextrose Therapy in Everyday Practice, New York, 1937.

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[13] Oelkers, H. A., and Schutze, G., *Klin. Wchnschr.*, 1938, **17**, 871.

TABLE II.
Course of Subdiabetic Rabbits.

Animal	Date of last alloxan	Test date	Glucose tolerance test type	Blood sugar level in mg/100 cc Hr 0	1	2	3
No. 2	10/22	1/6	Ordinary	84	119	104	99
		11	A.C.	113	122	120	111
No. 4	10/15	11	Ordinary	88	136	115	102
		13	A.C.	125	221	186	184
No. 5	10/28	6	Ordinary	—	276	247	211
No. 6	12/7	13	Ordinary	88	99	92	99
		18	A.C.	90	164	129	104

proportion to body weight seems unnecessary.[11]

As expected[12,13] the one hour blood sugar levels, while high, showed wide variation. By 2 hours, however, the means of the ordinary glucose tolerance tests had returned to the levels of the fasting means. Sole exception was the mean of the initial tolerance tests, where the 2 hour mean was slightly (15.2 mg%), but not significantly, elevated. This variation is due principally to Rabbits 4 and 8 which later exhibited normal glucose tolerance curves; excitement may account for the form of their initial curves.[15] The return to normal by 2 hours agrees with the previous observations.[2,11-13]

At 3 hours the means of all of the ordinary glucose tolerance tests approximated the fasting means. In occasional rabbits there was a marked rebound from the 2 hour level, amounting to as much as 40 mg%.

Modification of Glucose Tolerance by A.C. The effects of adrenal corticoids[16] and of pituitary adrenocorticotrophic hormone (A.C. T.H.)[17,18] on carbohydrate metabolism are well known. Adrenal cortex extract was chosen for the present experiments, rather than A.C.T.H, merely because it was immediately available. In selecting the dosage the aim was to use a quantity of hormone large enough to modify carbohydrate metabolism, yet small enough so that the glucose tolerance of a normal rabbit would be unaltered. The dose used, ½ cc/100 g body weight, is about 15% of the amount of A. C. which produces hyperglycemia in rats.[19] The preparation used was assayed by the manufacturer as containing "not less than 2.5 rat units/cc", and presumably it contained not much more. From Kuizenga's data[20] it may be calculated that the dosage of 11-dehydro-17-hydroxycorticosterone was approximately 0.04 mg/100 g body weight; this is about 2½% of the dose of corticoid which produces frank hyperglycemia in normal rats.[21]

The choice of dosage proved fortunate, since it did not modify the mean blood sugar levels of 4 rabbits subjected to A.C. tolerance tests prior to the administration of alloxan, while there was significant elevation of the mean 2 hour blood sugar level of the 6 alloxan treated rabbits subjected to A.C. tolerance tests. Since these alloxan treated rabbits had normal ordinary glucose tolerances, the A.C. tolerance test was effective in disclosing im-

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16 Long, C. N. H., in Duncan, G. G., Diseases of Metabolism, 2nd ed., Philadelphia, Pa., 1947.

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19 Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology*, 1940, **26**, 309.

20 Kuizenga, M. H., in Ingle, D. J., The Chemistry and Physiology of Hormones, A.A.A.S. monograph 21a, 1943.

21 Ingle, D. J., *Endocrinology*, 1941, **29**, 649.

paired carbohydrate metabolism not demonstrated by usual methods. As would be expected, a rabbit with diabetic glucose tolerance (No. 8) showed further impairment of its tolerance when pretreated with A.C.

Alloxan Subdiabetes. In these experiments one or two small doses of alloxan have produced a subdiabetic state in rabbits: namely disturbances in sugar metabolism which are so slight that they are not associated with either hyperglycemia or abnormal glucose tolerance. These slight physiologic disturbances have been demonstrated, in every instance, by the use of the A. C. tolerance test. This state is not discussed in the experimental literature, but it was probably produced by Shipley and Rannefeld.[1] These observers noted progressive and persistent impairment of glucose tolerance in rats injected with repeated 25 mg/kg doses of alloxan. In four of their rats, which had normal fasting blood sugars at the end of their alloxan courses, anterior pituitary extract produced a transient fasting hyperglycemia.

Three reports concerned with pancreatic islets suggest the presence of subdiabetes. These changes were reported in nondiabetic alloxan treated rats,[22] in 95% pancreatectomized rats prior to the onset of either latent or manifest diabetes,[5] and in one nondiabetic rabbit which received two 50 mg/kg doses of alloxan.[23] In each report the histologic findings were similar, namely hypertrophy and hyperplasia of the β cells in the larger islets, succeeded by β cell degranulation and degeneration, as few as 1/5 of the islets could be involved. It seems not unlikely that comparable changes occurred in the present experiments.

The course of subdiabetes in these rabbits remains under observation. Progression has been observed in one animal, and maintenance of the subdiabetes for as long as 15 weeks has been the rule in the others. It would not be surprising if remission ultimately occurred in some animals, since spontaneous remission of manifest alloxan diabetes has been reported.[24,25] None of the morbid anatomical changes seen in human diabetes mellitus were found in the three animals thus far autopsied.

Summary and conclusions. 1. Small doses of adrenal cortex extract (A.C.) do not modify the glucose tolerance of fasted normal rabbits.

2. One or two small doses of alloxan (less than 40 mg/kg) do not produce more than transient changes in the blood sugar level or glucose tolerance of normal rabbits.

3. Six rabbits, so treated, showed a significant impairment of glucose tolerance when pretreated with A.C. (A.C. tolerance test); the hormone effect was transient.

4. By definition the treated rabbits had alloxan subdiabetes: they had impaired carbohydrate metabolism unaccompanied by fasting hyperglycemia, glycosuria, or impaired ordinary glucose tolerance.

5. Alloxan subdiabetes persisted in the rabbits for the duration of the experiment (up to 15 weeks).

6. A hormone sensitized glucose tolerance test, such as the A.C. tolerance test, should prove useful in studying problems in human subdiabetes and prediabetes.

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17269. A Self-Contained Method for the Administration of Fluids at Regular Rates.

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The continuous administration of fluids has been of considerable importance in the study of many phases of physiological phenomena.

Certain critical limits to any system for such administration must be adhered to: (1) the rate of delivery must be linear over the range demanded by the experimental procedure; (2) the system must be reasonably foolproof and simple to adjust; and (3) the system should be compact.

For some experimental purposes a rate of flow, constant within 0.1% is necessary, and methods have been designed to meet such tolerances.[1,2] Such systems are susceptible to mechanical failure, entail considerable supervision, and though modified[3,4] to allow movement of the subject, necessitate a continuous connection between the subject and some stationary structure, not always easy to maintain for long periods in the experimental animal.

For purposes less rigorous in requirements of tolerance of rate of flow, a method has been devised with possibilities of adaptation to a multiplicity of experimental uses, allowing complete freedom of movement to the recipient of the fluid.

Construction. A glass container of suitable volume is fitted with a 2 hole rubber stopper carrying 2 short lengths of glass tubing, one of which is flanged at the lower end. Over this flanged end which lies within the container, is fitted a short length of latex rubber tubing fastened securely with silk thread. The free end of this latex tubing is also tied securely. To the second length of glass tubing is affixed a fine wire long enough to reach to the bottom of the glass container when the stopper is affixed (Fig. 1). The wire prevents tight sealing of the inflated tube to the glass wall.

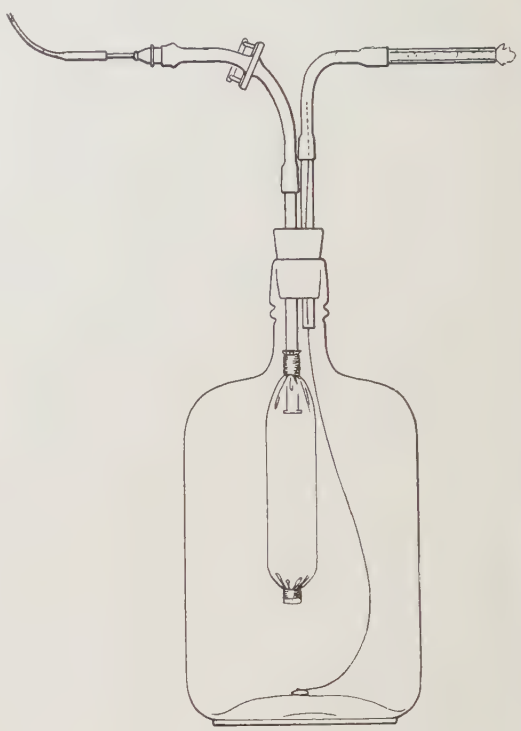

Fig. 1.

A typical delivery system shows the space relationships and mode of construction. The component parts are described in the text.

Operation. Filling. Two short lengths of rubber tubing are attached to the outer ends of the glass tubing perforating the stopper, and the stopper is fitted tightly into the neck of the glass container. Vacuum is then applied to the tube to which the wire has been attached, while the end of the tubing carrying the latex container is connected to a reservoir of fluid with which the apparatus is to be

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TABLE I.
Typical Systems.

Size of reservoir tubing	Delivery pressure, mmHg	Length of reservoir tubing, cm	Size of polythene tubing of delivery unit: Large (needle No. 18)	Medium (needle No. 18)	Small (needle No. 23)
			Delivery rate, cc per min.		
Infusion	155	100	20	4.5	.8
Aged 1 wk.		50	22	11	1.2
Single 3/8"	16	100	5	.8	.1
Aged 1 wk.		50	8	2.1	.2
Single 5/8"	14	100	3.5	.3	.08
Aged 1 wk.		50	5	1	.1
Double 5/8"	27	100	7	1.2	.15
Aged 1 wk.		50	10	2.1	.3
Double 1"	49	100	11	3	.4
Aged 1 wk.		50	18	5	.9

"Infusion" tubing—Rubber Latex Products, Inc., Cuyahoga Falls, Ohio.

All other rubber tubing—"Penrose" tubing from Davol Rubber Co., Providence 2, R. I. They have recently provided us with heavier 1½" tubing which has a delivery pressure of 41 mm single thickness and 70 mm double thickness, with comparable intermediate delivery rates.

Polythene tubing was supplied by Suprenant Electrical Insulation Company.

Large — 0.30 cm inside diam., 0.48 outside diam.
Medium—0.23 " " " 0.38 " "
Small — 0.11 " " " 0.24 " "

filled for delivery. The latex container is gradually distended with fluid, as the vacuum is created in the glass container, until it completely fills the bottle. The connection leading to the latex bag is then clamped off, and the apparatus is ready for delivery.

Delivery. A delivery unit of a suitable length and size of polythene tubing is connected to the clamped off tube connected to the latex bag. The needle or cannula of the delivery unit is inserted into the blood vessel or space desired and the clamp removed.

Delivery Rate. Reproducibility. The delivery rate of most of the fluid (60 to 80%) is linear, and for any given unit is constant within reasonable limits (±3%) on successive runs with the same resistance and with the same solution being delivered. Similar apparatus made of similar materials will reproduce rates within 6%. Ageing of the tubing in contact up to 2 weeks with the solution to be delivered produces no significant change in delivery rate with such inert solutions as physiological saline and low concentrations of organic compounds of intermediate acidity and basicity. Treatment with depyrogenizing agents such as weak bases (bicarbonate, sodium nitrite, green soap) had no effect on the delivery rate over 12 hours, but autoclaving drastically decreased the elasticity of the latex and affected the delivery rate. Sterilization for 12 hours with organic bactericides (Zephiran, Metaphen), however, had no noticeable effect.

Applications. This type of delivery unit is adaptable to many uses within a wide range of delivery rates (Table I). The desired delivery pressure may be roughly approximated by the selection of the type of tubing used in constructing the delivery unit. By using one latex container within another the arithmetic sum of their delivery pressures can be utilized. Delivery rate can be controlled most simply by increasing or decreasing the length of plastic tubing used for delivery. The rate may be slowed by increasing the viscosity of the solution, or by limiting the size of the air intake. If a relatively high viscosity fluid be admitted instead of air, the rate of delivery will be regulated by the rate of flow of the high viscosity fluid. To increase the rate of delivery, the pressure on the intake side may be increased by connecting two units in series. The latex containers

need not be similar in volume, expansion, or degree of elasticity. With solutions which react strongly with rubber, such a series system can be applied to deliver fluid at a given rate from an inelastic reservoir made of cellophane or viscose tubing.

Variations in pressure on the intake side of the apparatus can be made to regulate delivery to any given percentage by proper selection of the total pressure within the system. With thin walled tubing, variations of pressure up to 50% are in the physiological range. Thus, where the total delivery pressure is 50 mm Hg, variation of 10.0 mm in the circulatory system would cause a change of 20% in the rate of delivery. If however, the delivery pressure is 1200 mm, the variation in rate due to this will be less than 1%.

The apparatus is superior to gravity drip in several respects. It can be placed in a light canvas harness on an experimental animal to deliver fluids over a long period during which the animal is at complete liberty. Delivery rates are variable over a range from 20 cc per minute to 0.08 cc per minute with only a minor change in the rubber stock used for the inside container.

Summary. 1. Various combinations of self-contained delivery units for the administration of fluid at regular rates are described, and the limits of usefulness of these units are delineated.

2. Combinations of such units to permit the administration of various types of fluids, including those likely to destroy the elasticity of rubber, are described.

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17270. Apparatus for Cross Transfusion.

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Cross transfusion, the exchange of blood between a donor and a patient, which results in continuous mixing of the blood of two bodies, may be used in conditions such as uremia, resulting from acute renal insufficiency from a variety of causes, and in the treatment of other non-transmissible diseases.

The ideal requirements of a method for successful cross transfusion are: 1. Exchange of blood at a rate which may be varied from 0.5 to 10 liters per hour. 2. No chemical changes or hemolysis occurring in the transmitted blood. 3. Accurate measurement of the transferred blood. 4. Automatic equalization of the amounts of blood transferred in both directions. 5. The use of venous blood only, because donors and patients cannot be expected to sacrifice arteries every time that this procedure is applied.

In the past, cross transfusion has been applied in a variety of ways, none of which has been completely satisfactory. The method of paired arterial-venous anastomosis does not permit measurement of the exchanged blood and is obviously unsuitable. The multiple syringe method and the bottle method are tedious, slow and prone to introduce airborne infections. Pumps which operate on the "leaky valve" principle exhibit fluctuations in output which are proportional to the resistance encountered by the outflow. The worm pump (Pennell) and the roller pump (DeBakey) methods hemolyze at least 0.1% of the transferred blood and do not measure the transferred blood with sufficient accuracy. Although 0.1% of hemolysis is a tolerably small amount for ordinary direct transfusions of about 500 cc, yet, when quantities up to 50,000 cc are involved, the amount of hemolysis caused by such pumps may prove injurious.

This report deals with a new device which satisfies the above criteria for a safe, efficient

* Assisted by Dr. Joseph Miller. Supported by a grant from Mr. Emil Friedlander.

and easily managed method for cross transfusion.

The device consists of 4 units. 1. Suitable double lumen intravenous plastic cannulae. 2. A motor driven blood pump. 3. Bubble traps. 4. Metering and equalizing unit.

1. *The intravenous catheter.* Blood is withdrawn from and administered to the patient and donor through specially made intravenous catheters. These consist of extruded plastic double lumen tubes of an oval outer shape with an inside diameter of 2.5 mm for each conduit. The outside diameters of the catheters are 2.8 x 4 mm. The tip of the catheter is such as to provide for an outflow end protruding about 2″ from the intake. The tip of the catheter is equipped with a thin finger-like projection which serves as a guide, the end of which terminates in a round, plastic-coated lead shot. The intake opening is fashioned with an overhanging lip which tends to prevent occlusion of the intake opening by the walls of the veins, by valves or by clots. The catheter is inserted into a vein and is pushed cardiad until the tip lies in the superior or inferior vena cava. The intake opening is upstream from the output opening and therefore no blood which is discharged through the output opening is aspirated through the intake opening when the rate of flow in the vein exceeds the rate of intake into the catheter. In our experience, this catheter has performed well. It carried an adequate volume of blood. Clotting is prevented by the intravenous injection of 1-2 mg heparin per pound of body weight and the catheter may be left *in situ,* without excessive reactions, for days at a time.

2. *The pump* (Fig. 1) propels blood by applying negative pressure to the catheter intake channel and imparting a positive pressure to the withdrawn blood. A simple commercially available pure gum rubber tube (1) is laid in a receiving channel and is then raised to connect with the outside valves (2,4) and compression unit (3). The valves and compression unit are actuated by a motor driven shaft (5) which is ground to provide cams (6) and eccentrics (7) which operate the valves and the compression unit respectively. In this manner when the "intake"

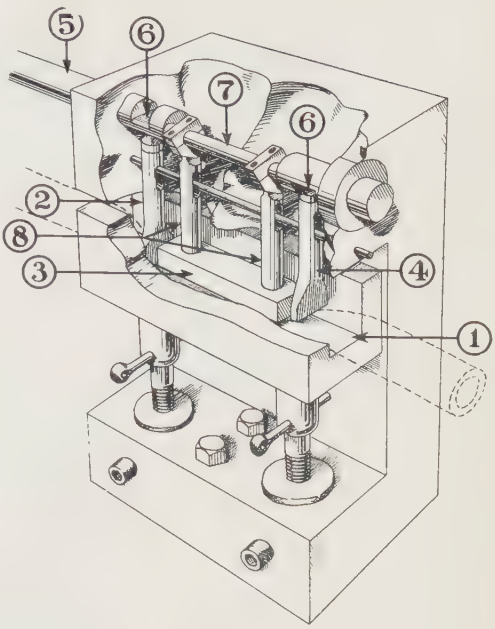

Fig. 1.
Blood pump.

valve (2) is "down" the "outlet" valve (4) "up" and the compression bar (3) comes down, the tube will be compressed and blood will be forced forward. When the "outlet" valve is "down" the "intake" valve "up" and the compression bar is raised, blood is aspirated into the tube. The tube is occluded only at the relatively short segments where it is compressed by the valves; the excursion of the compression unit is adjusted to compress the tube, but not to squeeze it. This precaution has eliminated hemolysis. The pump requires a minimum of attention, such as an occasional lubrication of the moving parts with glycerin. It is possible to change the stroke volume by using tubes of different sizes and by raising or lowering the receiving channel. The minute volume can also be controlled by adjusting the speed of the drive shaft and can be adjusted to between 10 and 1000 cc per minute.

The pump used here consists of 2 identical devices, mounted in opposite directions, of equal dimensions, and adjustment which provide an excellent means of effecting cross transfusion of equal amounts of blood. Small gas bubbles may form occasionally in the

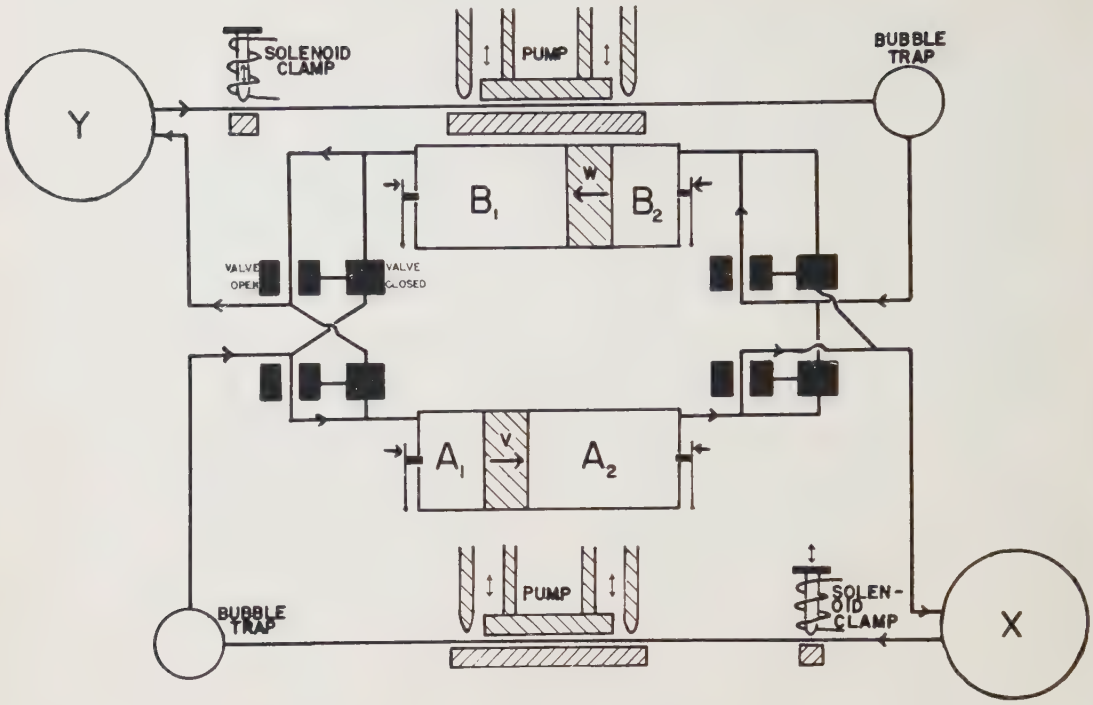

FIG. 2.
Diagram of blood flow in the cross transfusion apparatus.

blood because of the negative pressure exerted by the pump when it is withdrawing blood from the vein. Also, variations in stroke volume of the pump may occur because of changes in pressure in the intake channel, which are possibly caused by a reduction of the effective size of the intake opening. These two sources of error are corrected by bubble traps and a measuring and equalizing unit described below.

3. *Bubble traps.* These consist of 2 upright, cylindrical plastic chambers, one inch in diameter and 3½″ in height. Blood flows into one end of a chamber, near the top, and flows out at the bottom, allowing any air bubbles contained in it to collect at the top. Near the top of the cylinder there is a flexible plastic membrane which forms the floor of another plastic chamber which is filled with air and transmits variations in pressure to a U-type mercury manometer. Just underneath the plastic membrane there is a vent-like opening, to allow for adjustment of the blood level within the bubble trap and for the introduction of liquids into the bloodstream.

This device removes all but the very smallest air bubbles, allows for the constant indication of pressure and the administration of anticoagulants, drugs, etc., to either bloodstream during the experiment.

4. *The meter.* (Fig. 2) The metering unit receives blood into cylindrical chambers A1 and B2 from bodies of the subjects X and Y. As the blood is pumped into chambers A1 and B2, two floating plastic pistons, V and W, move toward the opposite ends of the chambers and expel the contents of chambers A2 and B1 into bodies X and Y respectively. At the end of the excursion of the floating pistons a micro-switch mechanism provides for the activation of solenoid clamps which stop the flow of blood to whichever chamber has been filled. When both chambers are filled, a valve mechanism is moved by means of a motor. As soon as the movement of the valve-changing mechanism is completed the solenoid clamps are released and blood again

flows into the measuring chambers. Blood from animal X is is now pumped into chamber B1 instead of Chamber A1, thereby expelling into animal X the blood from animal Y which had been collected in chamber B2 during the preceding cycle. The number of changes of the valve mechanism is recorded automatically on a meter and provides an easy and convenient way of determining mean blood flow and total amount exchanged. The volume of the chambers is 50 cc each.

This arrangement has the following advantages: It measures and equalizes the flow of blood in both directions automatically. It provides for simultaneous withdrawal and administration of equal amounts of blood. The accuracy of the registration of blood flow is about ± 1-3 cc in 10,000 cc, depending upon the fit of the piston and on the pressure differential in the two circuits. The device has been found completely satisfactory in experiments involving the exchange of a total, to date, of 500,000 cc of blood in both animals and human beings. The amount of heparin needed to prevent coagulation is within the clinically acceptable limits. Hemolysis does not occur if the pump is properly adjusted. Cross transfusion itself has proved to be a safe, efficient and relatively easy procedure, when performed with the apparatus described above. No fatalities attributable to the procedure itself have occurred.

Summary. A safe, effective and easily controlled apparatus for cross transfusion is described.

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17271. Vasomotor Reactions in the Mesenteric and Serosal Capillary Bed During Fright and Violent Muscular Activity.*

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Recently, technics were devised for painless exposure, with local anesthesia, of the guinea pig mesentery and gut and its maintenance in a warmed chamber where the capillary bed could be observed continuously with the microscope.[1] This permitted study of peripheral vasomotor reactions, and vascular responses to drugs without possible variables introduced by general anesthesia. In addition, the fact that the animal studied was fully conscious afforded the opportunity to witness the behavior of terminal arterioles and capillaries in this splanchnic area during various emotional states and spontaneous muscular activity.

Methods. Studies were made on 20 animals receiving a normal well supplemented diet, and on 21 animals in moderately advanced Vitamin C deficiency after 3-4 weeks on a standard scorbutogenic diet. The apparatus and diet have been described previously.[1] In brief, it includes holding a trained animal under light restraint, with cautious painless exposure of the mesentery and gut via a denervated or procaine field block in the flank. The preparation is maintained in a suitable warm chamber and continuously bathed with a drip solution of warmed buffered Ringer-gelatin solution, while being observed under the microscope.

Results. A. Animals receiving a "normal" diet. The changes found in the capillary bed of this splanchnic region in the control animals could be divided into 2 major categories. The

* This study was supported in part by a grant in aid from the Nutrition Foundation to Syracuse University College of Medicine.

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1 Lee, Richard E., and Lee, Nina Z., *Am. J. Physiol.*, 1947, **149**, 465.

terms "startle reaction" and "flight reaction" are based on the degree of overt manifestation of "emotional" disturbances.

1. The "startle reaction." It was first noticed that following a sudden loud noise, the animal being examined might shudder, or otherwise appear startled. The great majority of such responses were not accompanied by any observed phenomena in the splanchnic vascular bed. In approximately 20% of such situations, however, there was a slight narrowing of mesenteric and serosal arterioles of diameters ranging from 50-80 μ with general slowing of capillary blood flow. Precapillary sphincters usually narrowed partially. This vasoconstriction generally began 1-2 seconds after the brief startle response by the animal, and lasted 5-10 seconds at most. It frequently involved isolated regions of the arteriole, producing a band-like constriction of 30-40 μ in length, in which the arteriolar diameter was reduced occasionally by 25-50%. As repeated "startle" responses were induced in the same animal, it was noted that these sphincterlike constrictions of the arterioles invariably occurred at the same locus. Apparently, certain "key sites" along the small peripheral vessels, perhaps associated with the terminals of particular nerve fibers, are capable of response at levels of psychic stimulation below those required for a more generalized vasoconstriction.

2. The "flight reaction." Occasionally, the auditory and other stimuli would be followed by a much more vigorous and prolonged sudden reaction, in which the animal would struggle violently to escape from his bonds. This muscular effort (and apparently emotional disturbance) usually lasted for 5-20 seconds, then ceased as suddenly as it had begun. Motion of the fields under observation often prevented accurate visualization during these episodes.

Of those instances in which the mesentery remained relatively undisturbed, approximately 25% were accompanied by no observed vascular changes. In 50% of the episodes, vascular phenomena were found resembling those of the startle reaction but generally of greater magnitude. The remaining 25% of the "flight reactions" were accompanied by profound changes in the vessels. Within 1-2 seconds after the onset of struggling the "key sites" of the larger arterioles became greatly narrowed to completely occluded. Apparently at the same time, narrowing occurred in the entire remainder of the arteriolar components, with marked to complete constriction of all precapillary sphincters in the mesentery and serosa. This intense and widespread vasoconstriction resulted in a complete cessation of observed arteriolar blood flow to the gut, with limitation of active circulation to the larger mesenteric arterial arcades. The blood flow in the venules and veins became very sluggish, but continued. This afforded a relatively rapid drainage into the general circulation of much of the residual capillary blood.

This picture of complete capillary stagnation lasted throughout the period of excitement and muscular activity. When struggling ceased, frequently no change was seen for 5-10 seconds. The first areas to release their spasm were the "key sites" along the larger arterioles. Flow gradually increased through the narrowed arteriolar tree along the more direct A-V channels. Within 15-20 seconds, the smaller arterioles began to dilate, resulting in augmentation of flow in the arteriolar net. It was frequently 1-2 minutes, however, before sufficient pre-capillary sphincters had opened to permit a return of adequate capillary circulation. Following the disappearance of this vasospastic state, no further obvious changes were noted in this study.

In 3 instances, observations on arteriolar and precapillary sensitivity to topical epinephrine were made immediately after the resumption of a normal capillary flow. In each it was found to be at least 300% greater than control readings of 1:2,000,000. Five minutes later it had returned to normal values.

B. The scorbutic animals. Although the "startle responses" and the "flight responses" occurred as frequently and as readily in the scorbutic guinea pigs as compared to the pair-fed controls, circulatory changes during or following these episodes were not observed. The relatively depressed condition of the peri-

pheral vascular system in this nutritional deficiency, and its possible significance has been reported previously.[1]

Discussion. Although the mucosal blood vessels were not observed directly in this study, the complete cessation of blood flow found frequently in the small arteries and arterioles supplying the mesentery and traversing it to supply the intestine support the assumption that accompanying the "flight reaction" there is often a complete stagnation of blood flow in this viscus. This is apparently maintained throughout the period of response to the stimulus, and subsequently returns slowly. Of particular interest is the fact that even at the height of response, continuous blood flow through the arterial arcades and the larger venules and veins in the mesentery affords a means of draining much of the residual capillary blood in this region and returning it, along with the blood shunted to the arcade system, back to the general circulation. One can assume that this would considerably increase the volume of blood available for other somatic areas during the period of active vascular response.

The absence of vasospasm in the mesentery and serosa during the flight response in scorbutic animals is associated with a general depression of arteriolar and precapillary tone, and impaired venular flow.[1] It remains to be determined whether these manifestations of ascorbic acid deficiency are a result of general tissue disfunction or are perhaps a specific defect in some vasotonic mechanism.

Summary and conclusions. 1. Technics were devised for viewing the mesenteric and serosal capillary bed of guinea pigs not under a general anesthetic agent.

2. The degree of emotional response to auditory stimuli could be correlated with the magnitude of changes in the visualized splanchnic vessels.

3. (a) A "startle response" was occasionally accompanied by transient slight vasoconstriction and slowing of blood flow.

(b) A "flight response" was frequently characterized by intense vasoconstriction of arterioles, complete stagnation of capillary blood flow, and a drainage of residual capillary blood into patent venules.

3. Certain "key sites" along the arteriolar tree have a lower threshold of response than remaining areas, and exert much control of arteriolar flow by sphincter-like activity.

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17272. Reversal of Insulin-Induced Hypoglycemia in Chick Embryos by Nicotinamide and α-Ketoglutaric Acid.*

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In a series of papers Landauer and his collaborators[1–4] have described the teratogenic effects which insulin produces when it is injected into the yolk sac of chick embryos. When the insulin is applied in this manner to early embryos (0-72 hrs) caudal defects (rumplessness) are the most frequently encountered anomalies. When injected into older embryos (96-168 hrs) the insulin causes a high incidence of disproportionate shortening of the legs (micromelia). These effects of insulin disappear when the insulin is inactivated and reappear when inactivated insulin is reactivated. Landauer[5] demonstrated that when

* This investigation was aided, in part, by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

1 Landauer, W., *J. Exp. Zool.*, 1945, **98**, 65.

2 Landauer, W., and Lang, E. H., *J. Exp. Zool.*, 1946, **101**, 41.

3 Landauer, W., and Bliss, C. I., *J. Exp. Zool.*, 1946, **102**, 1.

4 Landauer, W., *J. Exp. Zool.*, 1947, **105**, 145.

5 Landauer, W., *J. Exp. Zool.*, 1948, **109**, 283.

nicotinamide is injected into the yolk sac either shortly before, after or simultaneously with insulin the incidence of micromelia is reduced from 41 - 53% to 3 - 9% depending on individual experiments. When the same experiments were repeated on earlier stages it was found that nicotinamide also reduces the incidence of rumplessness. In like manner α-ketoglutaric acid reduces the incidence of micromelia, though not as effectively as nicotinamide, but does not, however, exert much of an inhibiting effect on the rumpless-inducing properties of the insulin.

Zwilling[6] showed that the injection of insulin into the yolk sac of 120 hr embryos is followed by an hypoglycemia which, in some cases, persists for 8 days. The blood sugar in all embryos is restored to normal levels by the fourteenth day of development. In the course of this investigation it was found that a very good correlation exists between the degree and persistence of the hypoglycemia and the degree and incidence of micromelia. Factors which increase the micromelic effects (such as adrenal cortical extracts, Landauer[7]) also exaggerate the hypoglycemic effects.

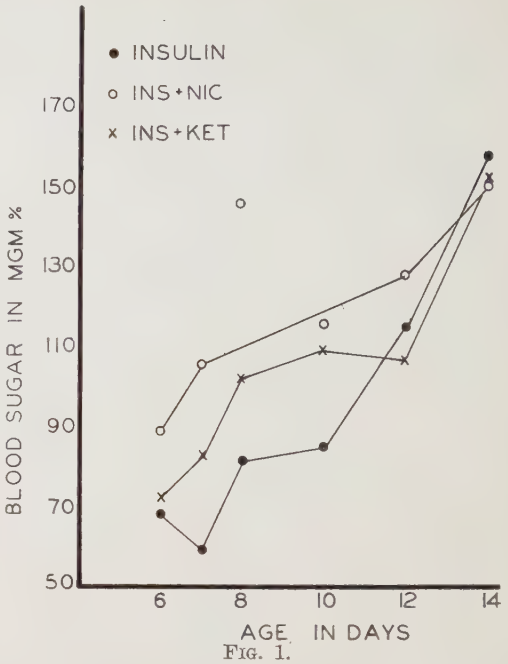

FIG. 1.

Course of blood sugar in chick embryos treated with: insulin alone (2 units), insulin + nicotinamide (18.9 mg), and insulin + α-ketoglutaric acid (20.2 mg) at 5 days. Each point represents the average of blood sugar values for all embryos assayed at a given stage.

It was of some interest to us to determine whether nicotinamide and α-ketoglutaric acid have any effect on the insulin-induced hypoglycemia. We found that these substances do decrease the incidence of hypoglycemia and that this decrease follows the same order that it does for their micromelia-alleviating capacities; *i.e.* the effect is more pronounced with nicotinamide.

Material and method. Eggs from unselected White Leghorn hens were used in this work. They were all injected at 120 hours as described by Landauer.[1] When two substances were used they were injected separately, but in sufficiently rapid succession so that they may be considered simultaneous injections. Blood was obtained and assayed for reducing substances by the technic described previously.[6] For each stage blood samples were taken from embryos which had been injected with insulin alone (2 units), nicotinamide alone (18.9 mg) or α-ketoglutaric acid alone (20.2 mg) and both insulin and one of the other two substances. The sequence of obtaining blood from the embryos was altered sufficiently so that it could not influence the results.

Results. The data from these experiments are presented in Fig. 1 and in Table I. Since neither nicotinamide alone nor α-ketoglutaric acid alone cause any marked deviation from normal blood sugar levels the data obtained for these substances have been omitted. All of the points on these curves represent averages for all blood sugar values obtained for a given stage. This explains the difference between the curve for 2 units of insulin presented here and that presented in the previous paper.[6] In the latter the points represented the average values for all hypoglycemic embryos at any given stage. However, these data for insulin alone duplicate those of last year in all essential respects. It can be seen that both nicotinamide and α-ketoglutaric

[6] Zwilling, E., *J. Exp. Zool.*, 1948, **109**, 197.

[7] Landauer, W., *Endocrinol.*, 1947, **41**, 489.

TABLE I.

Summary of Blood Sugar Data from Embryos Injected with Insulin (2 Units), Insulin + Nicotinamide (18.9 mg) and Insulin + α-ketoglutaric Acid (20.2 mg). All injections were into the yolk sac of 5 day embryos. In the last column under each substance the range of blood sugar values is given for only the hypoglycemic embryos. Values in the normal range have been omitted.

	2 units insulin			Insulin + nicotinamide			Insulin + α-ketoglutaric acid		
Age in days	No. of embryos	No. hypoglycemic	Range of hypoglycemia, mg %	No. of embryos	No. hypoglycemic	Range of hypoglycemia, mg %	No. embryos	No. hypoglycemic	Range of hypoglycemia, mg %
6	13	13	43.4- 97.2	10	9	62.5-108.6	10	9	42.7- 83.9
7	13	13	38.0- 85.3	9	4	67.8- 90.0	10	9	59.4-103.0
8	16	15	49.8-101.2	10	2	100.5-105.6	18	11	61.3-105.0
10	16	13	40.0-105.1	11	4	66.9- 96.1	18	10	43.1-108.2
12	13	6	34.7- 95.8	9	2	42.4- 94.0	8	4	32.1- 88.2
14	7	0		6	0		7	0	

acid reduce the average hypoglycemia and that this effect of the nicotinamide is evident within twenty-four hours of the time that it is administered. From the data presented in Table I it is evident that this is due, in large part, to a reduction in the number of embryos which are hypoglycemic at any one time. However there is also a decrease in the degree of hypoglycemia of the individual embryos. Nicotinamide is more effective than α-ketoglutaric acid in producing both of these results. This correlates well with the fact that cases of extreme micromelia are not found following injection of insulin + nicotinamide, while they do occur after injection of insulin + α-ketoglutaric acid. The one discordant point (insulin + nicotinamide at 8 days) may be due to a sampling error. However, there is a possibility that it may, in part, represent an actual increase. Not only were there fewer cases of hypoglycemia but the range of "normal" values was higher than usual. A similar phenomenon has been observed in our other work; it seems as though recovery to normal levels may be accompanied by an over-compensation.

Discussion. Again we have an instance in which the level of blood sugar varies as does the degree of micromelia. In this instance nicotinamide and α-ketoglutaric acid are instrumental both in decreasing the incidence of micromelia and in restoring the blood sugar of the embryos to more normal levels. It might be pointed out that the curve for insulin + nicotinamide described here is very similar to the one which describes the course followed by the blood sugar in embryos which receive only insulin but which show none of the teratogenic effects of that substance.[6]

The causal relationship between the morphological effects of insulin and its hypoglycemic action in the chick embryo is still obscure. It is not yet certain that these effects are *directly* due to a decrease in available carbohydrate energy, though it is evident that the carbohydrate metabolism is involved. In like manner the mechanism of the alleviating action of the nicotinamide is not clear. It may either be a result of its restoring the blood sugar to normal levels or due to a more direct action on the embryonic tissues.

We have found that sulfanilamide, which produces essentially the same micromelia as insulin,[8] does not alter the blood sugar (unpublished). However its micromelic effects are also reversed by nicotinamide (unpublished). There is a possibility that the low concentration of reducing sugars in the insulin-treated embryos may result in a higher nicotinamide requirement, while the sulfonamide may affect the nicotinamide content in another fashion. Levy and Young[9] have shown that virtually all of the nicotinamide (or nicotinic acid) in the chick embryo is bound in the diphosphopyridine nucleotide (coenzyme I). They also indicate (as has been found in other animal tissues[10]) that excess nicotinic acid (or amide) does not result in an increase in the coenzyme beyond its maximum levels. If these same conditions obtain under the abnormal circumstances induced by insulin (and sulfanilamide), then it seems likely that these substances may interfere, in some manner, with the synthesis of the coenzyme.

The hypoglycemia-alleviating effect of nicotinamide demonstrated here is not without precedent. Burke and McIntyre[11] have shown that this substance increases the tolerance of rats to insulin; a decrease in the duration of hypoglycemia was noted following the injection of a standard dose of insulin into nicotinamide-treated animals. These are essentially the same results which we have obtained with chick embryos.

That α-ketoglutaric acid also prevents insulin abnormalities as well as decreasing the duration of the hypoglycemia may indicate that pyruvate metabolism is, in some way, inhibited (possibly via coenzyme lack) and that this alternate pathway may be utilized as an energy source. At any rate these data indicate that carbohydrate metabolism is of considerable importance for normal limb formation in chick embryos.

Summary. The duration of insulin-induced hypoglycemia is reduced following the administration of nicotinamide and α-ketoglutaric acid to insulin treated chick embryos. Nicotinamide, which is the more effective in preventing insulin-induced abnormalities, is also more effective in decreasing the duration of the hypoglycemia.

[8] Ancel, P., *Ann. d'Endocrinol.*, 1945, **6**, 1.

[9] Levy, M., and Young, N. F., *J. Biol. Chem.*, 1948, **176**, 185.

[10] Schlenk, F., A Symposium on Respiratory Enzymes, Univ. of Wis. Press, 1942.

[11] Burke, J. C., and McIntyre, A. R., *J. Pharm. and Exp. Therap.*, 1939, **67**, 142.

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17273. Influence of Methionine and Thiouracil on Nitrogen Balance Index and Organ Weights of Adult Rat.*

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Allison, Anderson, and Seeley[1] have demonstrated that small quantities of methionine conserve body nitrogen when added to the diet of the dog. Earle, Small, and Victor[2] and Reisen, *et al.*[3] have found, however, that larger amounts of the amino acid will cause a marked loss in body weight in rats. This is in apparent contradiction to Harrison and Long[4] who reported that a diet containing 2% of methionine would increase the liver nitrogen of rats. Lea-

* Presented as partial fulfillment of the degree of Doctor of Philosophy at Rutgers University.

† Gerard Swope Fellow of the General Electric Company.

[1] Allison, J. B., Anderson, J. A., and Seeley, R. D., *J. Nutrition*, 1946, **33**, 361.

[2] Earle, D. J., Jr., Small, K., and Victor, J., *J. Exp. Med.*, 1942, **76**, 317.

[3] Reisen, W. H., Schweigert, B. S., and Elvehjem, C. A., *Arch. Biochem.*, 1946, **10**, 307.

[4] Harrison, H. C., and Long, C. N. H., *J. Biol. Chem.*, 1945, **161**, 547.

them[5] has found the same apparent contradiction in rats fed anti-thyroid drugs. The animals in his experiments lost weight, probably due to a restriction in food intake, but the protein content of the liver was remarkably increased. Leathem and Seeley[6] have also demonstrated that the plasma proteins of rats fed the anti-thyroid drugs were increased with the largest increase occurring in plasma globulins.

In an attempt to resolve these apparent contradictions, the following experiments were designed to determine the nitrogen balance at various levels of methionine and thiouracil in the diet and to determine the nitrogen content of the plasma and tissues.

Methods. Nitrogen balance indexes‡ were determined on adult rats of the Long-Evans Strain using the diets and technics previously described for the dog.[7] The diet contained 12% casein on a dry weight basis and the amino acid and thiouracil were added at various levels. Rats were pair fed with those receiving methionine (4.8%) where the food intake was markedly restricted. Experiments were also performed on animals fed *ad libitum* as a secondary control. The experiments were continued for 20 days with urine and fecal collections throughout the period. At the end of the experiment the animals were bled by heart puncture and autopsied. The plasma was analyzed by the method of Howe[8] as modified by Robinson, Price, and Hogdem.[9] The livers and kidneys were dried to constant weight at 95°C and were then analyzed for nitrogen by the micro-Kjeldahl procedure.

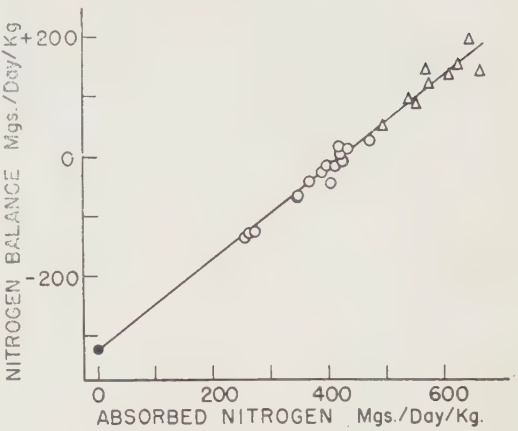

FIG. 1.
The relationship between nitrogen balance and absorbed nitrogen in rats fed a 12% casein ration *ad libitum* as indicated by the triangles, a 12% casein ration on a restricted food intake as indicated by the open circles, and a protein-free diet as indicated by the closed circle.

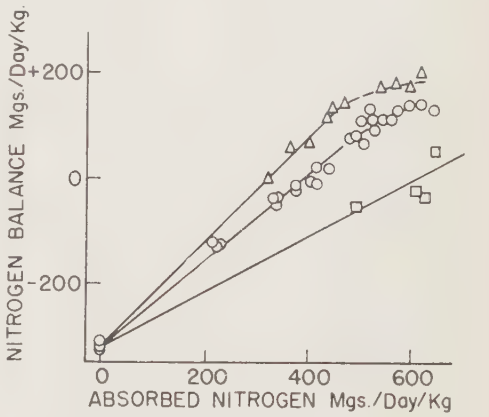

FIG. 2.
The relationship between nitrogen balance and absorbed nitrogen in rats fed a diet of 12% casein as indicated by the open circles, 12% casein to which was added 0.67% methionine (△), and 12% casein to which 0.6% thiouracil (□) had been added.

The urine and feces were also analyzed by the micro-Kjeldahl procedure. The Folin method[10] was used for the analysis of creatine and creatinine.

Results. The points plotted in Fig. 1 illustrate data obtained on rats fed a 12% casein ration (dry weight basis) *ad libitum* as shown by the triangles, and rats fed the 12% casein

[5] Leathem, J. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 203.

[6] Leathem, J. H., and Seeley, R. D., *Am. J. Physiology*, 1947, **149**, 561.

‡ The nitrogen balance index may be defined by the equation

$$NB = K(AN) - NE^{\circ}$$

Where NB is the nitrogen balance, AN the absorbed nitrogen, and NE° is the excretion of nitrogen on a protein free diet. Under these conditions, K may be defined as the nitrogen balance index.

[7] Allison, J. B., and Anderson, J. A., *J. Nutrition*, 1945, **29**, 413.

[8] Howe, P. E., *J. Biol. Chem.*, 1921, **49**, 93.

[9] Robinson, H. W., Price, J. W., and Hogdem, C. G., *J. Biol. Chem.*, 1937, **120**, 491.

[10] Folin, O. S., *J. Biol. Chem.*, 1914, **17**, 469.

TABLE I.

Excretion of Creatine in Rats Fed a Basal 12% Casein Diet to Which Was Added Varying Amounts of Thiouracil and Methionine. Experiments on a restricted food intake are followed by R and those in which the animals are fed *ad libitum* are indicated by A. The nitrogen balance index and the weight change over a 20-day period are included.

Dietary regime	No. of animals	Avg. wt. change 20-day period, g	N. balance index	Creatine, mg/day/kg
Control (R)	18	—17	.77	11.8 ± .3*
" (A)	14	50	.78	9.6 ± .3
Thiouracil				
.12 (R)	10	0	.85	8.7 ± .2
.67 (R)	8	—20	.58	5.6 ± .2
.67 (A)	12	+40	.58	3.0 ± .2
Methionine				
.67 (A)	10	56	.96	8.1 ± .1
.67 (R)	12	5	.96	9.0 ± .1
2.4 (R)	8	—40	.60	7.9 ± .1
4.8 (R)	14	—56	.60	9.2 ± .2

* $E = \sqrt{\frac{\Sigma d^2}{N\,(N-1)}}$ Note: This equation for the standard error applies to all figures in the tables for which such data are given.

ration on a restricted caloric intake as shown by the open circles. The relationship between nitrogen balance and absorbed nitrogen is linear throughout the region of negative and of low positive nitrogen balance with an average nitrogen balance index of 0.77.[11] The recent work of Bricker and Mitchell[12] has confirmed this relationship in the rat.

The nitrogen balance index of the casein may be altered by adding certain compounds to the diet. The data in Table I demonstrate that the addition of 0.67% methionine (dry weight basis) to a casein diet increased the nitrogen balance index of casein in the rat from 0.77 to 0.96. The addition of larger amounts of methionine, however, decreased the nitrogen balance index markedly. The larger doses of methionine caused a definite restriction of food intake and necessitated the paired feeding of other groups to the methionine group. This restriction in food intake is paralleled by the weight change shown in Table I.

Thiouracil also had a marked effect on the nitrogen balance index of the casein. The addition of 0.6% thiouracil to the casein diet gave a nitrogen balance index of 0.58. When .12% thiouracil was fed to rats on a restricted caloric intake, the nitrogen balance index was 0.85, and a value somewhat above that obtained with casein alone. Very high levels of thiouracil were not fed because Astwood[13] has reported that rats refuse diets containing high levels of the drug.

Despite the variation in nitrogen balance index produced by the addition of thiouracil or methionine to the casein diet, the straight line relationship between nitrogen balance and absorbed nitrogen is still valid. The data plotted in Fig. 2 demonstrate that the addition of methionine (0.6%) which increased the nitrogen balance index, and of thiouracil (0.6%) which decreased the nitrogen balance index did not alter the linearity of the relationship. The bending of the line at high nitrogen balance values is to be expected and has been reported by Allison and Anderson.[7]

The data in Table I demonstrate also that thiouracil decreases the excretion of creatine. The decrease in creatine excretion of the thyroidectomized animal has been reported by Allison and Leonard[14] and Wilkins and

[11] Brown, J. H., and Allison, J. B., Abstract 112th Meeting of Am. Chem. Soc., 1947, 51c.

[12] Bricker, H., and Mitchell, H. H., *J. Nutrition*, 1947, **34**, 491.

[13] Astwood, E. B., *J. Pharm. and Exp. Therap.*, 1943, **78**, 79.

[14] Allison, J. B., and Leonard, S. L., *Am. J. Physiol.*, 1941, **132**, 185.

TABLE II.

Data Obtained on Autopsy of Rats Fed a 12% Casein Diet *ad libitum* (A) and a 12% Casein Diet on a Restricted Food Intake (R) to Which Was Added Varying Amounts of Methionine Thiouracil.

Dietary regime	Thyroid, mg/100 g., B.W.	Liver, mg/100 g., B.W.	Kidney, mg/100 g., B.W.
Control (R)	6.7 ± 0.9	3230 ± 48	684 ± 24
" (A)	6.2 ± 0.5	3352 ± 52	691 ± 36
Thiouracil			
.12 (R)	9.5 ± 0.8	3450 ± 142	673 ± 32
.67 (R)	12.8 ± 1.2	3892 ± 210	684 ± 37
.67 (A)	16.2 ± 1.3	4221 ± 178	684 ± 31
Methionine			
.67 (A)	6.8 ± 0.7	2931 ± 96	811 ± 82
.67 (R)	6.4 ± 0.6	3193 ± 103	705 ± 40
2.4 (R)	7.2 ± 0.9	2961 ± 80	850 ± 32
4.8 (R)	8.7 ± 0.9	3290 ± 92	925 ± 46

TABLE III.

Influence of Various Concentrations of Methionine and Thiouracil on Liver Nitrogen of Rats Receiving a 12% Casein Basal Ration.

Dietary regime, sulfur	No. of animals	Liver N, %	Total Liver N, g/100 g., B.W.	H_2O, %
Casein (A)	18	9.2 ± .6	.098 ± .004	69.8 ± 1.2
" (R)	14	9.6 ± .4	.099 ± .003	71.2 ± 0.8
Methionine				
.67 (A)	10	9.7 ± .5	.107 ± .004	72.0 ± 1.3
.67 (R)	12	10.4 ± .4	.103 ± .003	72.7 ± 1.1
2.4 (R)	8	10.8 ± .1	.104 ± .003	71.1 ± 0.9
4.8 (R)	14	11.2 ± .6	.109 ± .004	70.3 ± 0.8
Thiouracil				
.12 (A)	10	8.6 ± .3	.091 ± .004	67.4 ± 1.0
.67 (A)	8	9.4 ± .3	.130 ± .010	69.5 ± 1.1
.67 (R)	12	9.3 ± .4	.106 ± .008	69.2 ± 1.2

Fleischman[15] have demonstrated that the creatine excretion of the thyrotoxic patient can be decreased by the administration of thiouracil. The data presented here demonstrate that the excretion of creatine is decreased in normal rats rendered hypothyroid by the administration of thiouracil. Methionine also demonstrated some tendency to decrease the excretion of creatine.

Thiouracil increases the size of the thyroid in rats fed the compound, (Table II). These data demonstrate that thiouracil not only increases the size of thyroid but also markedly increases the size of the liver. The increased size of livers in rats fed large doses of thiouracil has been previously reported by Leathem,[5] but his data was not correlated with a change in the nitrogen balance index.

Methionine, on the other hand, had no effect on the size of the liver but brought about a definite hypertrophy of the kidneys of rats fed high concentrations of this amino acid. Methionine in high level dosage also enlarged the thyroid. The enlargement of the thyroid upon the addition of methionine to the diet may be due to the high concentration of sulphur in the diet or to an antithyroid effect from the methionine. The changes in organ weights with both compounds are directly proportional to the dosage.

The nitrogen content of certain tissues of the body was altered by the addition of either

[15] Wilkins, L., and Fleischman, W., *J. Clin. Invest.*, 1946, **25**, 360.

TABLE IV.
Influence of Various Concentrations of Methionine and Thiouracil on the Plasma Proteins of the Rat Fed a Basal Ration Containing 12% Casein.

Dietary regime	No. of animals	Albumin G, %	Globulin G, %	A/G	Hematocrit, %
Control (A)	18	3.10 ± .14	2.89 ± .14	1.10	48
" (R)	14	2.96 ± .22	2.86 ± .16	1.14	48
Thiouracil					
.12 (R)	10	3.20 ± .18	3.73 ± .18	0.86	46
.67 (A)	8	2.97 ± .16	3.74 ± .19	0.68	43
.67 (R)	12	3.00 ± .14	3.48 ± .17	0.67	43
Methionine					
.67 (R)	10	2.86 ± .19	3.69 ± .21	0.78	44
.67 (A)	12	2.81 ± .16	3.77 ± .19	0.74	45
2.4 (R)	8	2.89 ± .22	3.69 ± .18	0.78	47
4.8 (R)	14	3.02 ± .23	3.65 ± .19	0.82	46

thiouracil or methionine to the casein diet. The data presented in Table III demonstrate that methionine increased the nitrogen per gram of tissue of the liver and produced an over-all increase in liver-nitrogen. Thiouracil also increased the total liver nitrogen markedly, but the increase was due to the increased size of the organ rather than to the increased nitrogen per gram of tissue. Both methionine and thiouracil increased the globulin fraction of plasma with little change in albumin and decreased the A/G ratio (Table IV).

Summary. The addition of methionine and of thiouracil to the diet of rats altered the nitrogen balance index in proportion to the level of the compound in the diet. Although both methionine in high concentration and thiouracil caused a decrease in body weight and a reduction in nitrogen balance index, the compounds increased the total liver nitrogen; however, methionine increased the nitrogen per gram of tissue without affecting the size of the organ while thiouracil increased the size of the organ without affecting the nitrogen per gram of tissue. Both methionine and thiouracil increased the size of the thyroid, while methionine alone increased the size of the kidney. Both compounds increased the total plasma proteins by increasing the plasma globulin. Both methionine and thiouracil decreased creatine excretion; however, the effect of thiouracil is marked in comparison to methionine. These data indicate that the result of feeding methionine or thiouracil is very similar in end result but that the mechanism may not be identical because the results are reached through different pathways.

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17274. Replacement of a Portion of the Common Bile Duct with a Segment of Uterine Horn.

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Attempts at repair are comparatively unsuccessful when the common bile duct is accidentally so injured that an end-to-end anastomosis is not feasible. The present studies were undertaken to ascertain whether a grafted tubular structure with independent blood supply would maintain its viability and carry the bile from the common bile duct to the duodenum. Dogs were selected for the experiments and the uterine horn was used for

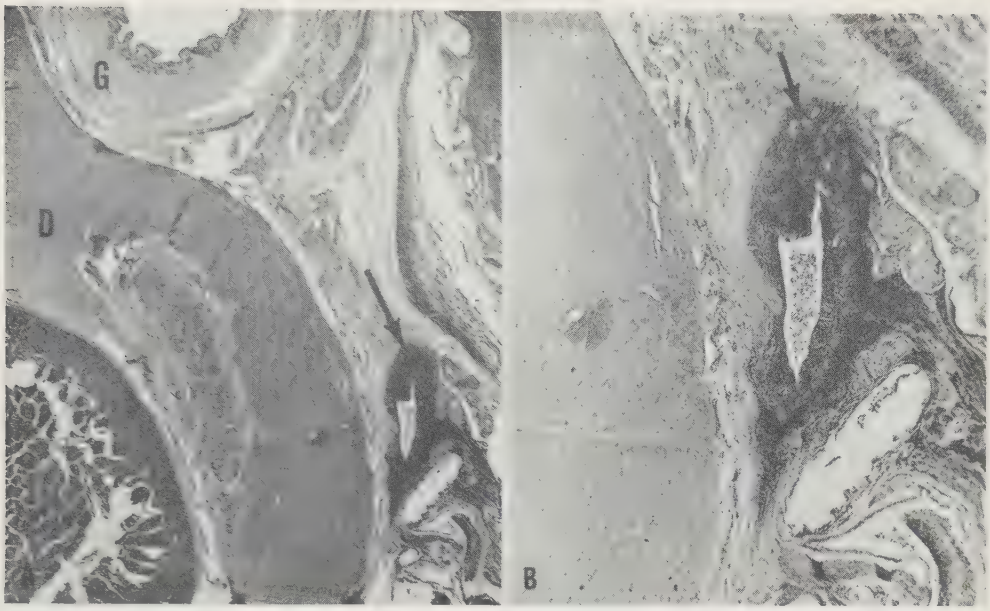

FIG. 1.
Duodenum (D), gallbladder (G), and transplanted portion of the right uterine horn (arrow) are seen from the dog 20 weeks following operation (A left) × 12. Cross section of the uterine horn enlarged (B right) × 20.

grafting.

Method. Under intravenous nembutal anesthesia the abdomen was opened with a midline incision, the right uterine horn was clamped, and its proximal end ligated. The uterine artery was also ligated and severed at this level. The "broad ligament" was then freed by ligating its blood vessels along its posterior margin. The proximal 5 cm of the uterine horn was freed from the distal segment by applying a ligature and cutting proximal to it, preserving its blood supply from the ovarian artery. The common bile duct was ligated near its junction with the duodenum and severed distal to the ligature. The cut end of the common bile duct was then telescoped into the proximal end of the uterine horn while the opposite end of the segment was inserted into the duodenal wall through a longitudinal slit made about 2 cm anterior and medial to the choledochoduodenal junction. Both ends of the graft were secured in place by cotton sutures. The abdomen was closed with through and through interrupted cotton sutures.

Of 20 dogs surviving the operation, 12 died in from 3 to 21 days. The remaining 8 dogs were sacrificed between the 4th and the 27th weeks following operation: 2 in the 4th week and one each in the 6th, 14th, 16th, 20th, 24th, and 27th weeks. The abdominal organs of the latter animals were placed in 5% formalin and after fixation were studied grossly and microscopically.

Results. None of the 8 dogs that were killed 4 weeks or later had clay colored stools, or any evidence of jaundice in the sclerae or viscera. In each dog there were fairly dense adhesions about the site of operation involving the liver, stomach, duodenum, great omentum, and loops of small intestine. Numerous blood vessels of somewhat increased caliber from the ovarian artery appeared to supply the graft. The surface of the liver was smooth, glistening, and of the usual color. The gallbladder was distended with bile, and pressure on the viscus yielded bile in the duodenum at the site of implantation of the uterine horn.

The intrahepatic biliary ducts had spacious lumina. The hepatic ducts could not be clearly exposed. The common bile duct continued imperceptibly into the segment of

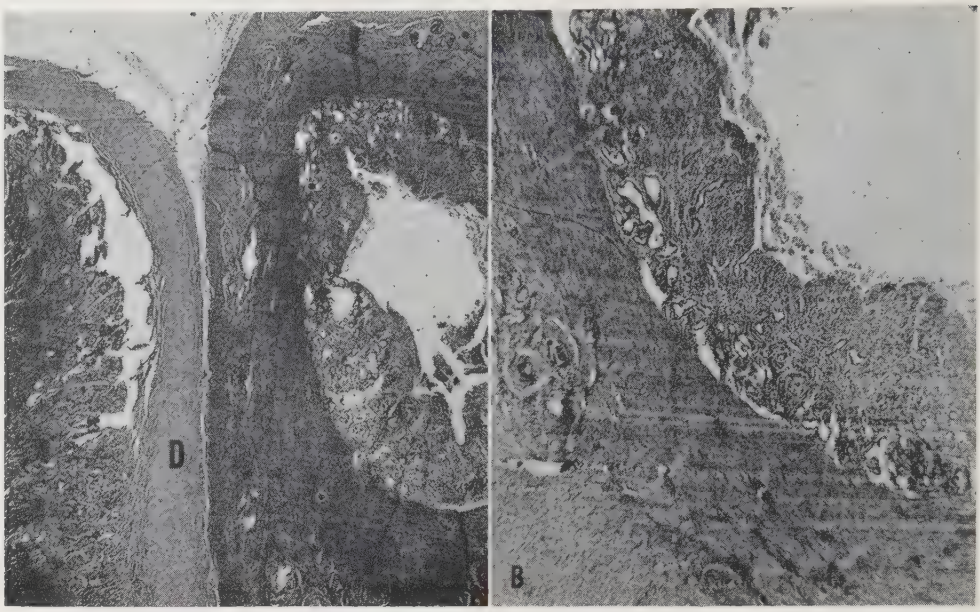

FIG. 2.
Duodenum (D) and transplanted portion of the right uterine horn are seen from the dog 6 weeks following operation (A left) × 12. Cross section of the uterine horn enlarged (B right) × 20.

uterine horn that connected it with the lumen of the duodenum.

After completion of the gross studies, microscopic sections were prepared of the liver, gallbladder, transplanted uterine segment, duodenum, and left uterine horn.

In the liver there were chronic cholangitis and pericholangitis, evidenced by an infiltration with lymphocytes, plasma cells, and large mononuclear cells in the wall of the bile ducts and in the surrounding portal connective tissue. The liver lobules were somewhat diminished in size and in their central portions there was some slight increase of the connective tissue and a decrease in size of the liver cells. Many of the bile capillaries contained inspissated secretion. In the gallbladders of some of the dogs there was slight to moderate chronic cholecystitis with focal and diffuse infiltrations of the tunica propria with chronic inflammatory cells. In all of the animals the implanted segment of the uterine horn carried the bile from the common bile duct to the duodenum. In some instances, the columnar epithelium covering the surface was almost intact. In others, the epithelium was intact only in the fundi of the glands and a cellular debris covered granulation tissue that partly replaced the bile stained mucosa. Invariably the muscular coats appeared intact (Fig. 1 and 2).

Comment. There is no submucosa in either the common bile duct or the uterine horn of the dog. Of the 2 the uterine horn is the sturdier structure. In the human, also, the common bile duct and the Fallopian tube have no submucosa. Of these the Fallopian tube is usually the more delicate structure. These experiments were conducted, not to suggest that the Fallopian tube be used as a pedicle graft, but rather to ascertain whether a grafted tubular structure with independent blood supply would serve for the passage of bile from the common bile duct to the duodenum. The evidence herein presented proved that this can be accomplished.

Summary. Experiments were conducted to ascertain whether a grafted tubular structure with independent blood supply would serve for the passage of bile from the common bile duct to the duodenum. Dogs were selected for the experiments and the uterine horn was

used for the grafting. Of 20 dogs surviving the operation, 12 died in from 3 to 21 days, and the rest were sacrificed between the 4th and the 27th weeks following the operation. Gross and microscopic studies of the biliary system of the latter 8 dogs disclosed that, in all, the transplanted segment of the uterine horn connected the common bile duct and the duodenum without causing permanent severe damage to the biliary system.

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17275. Observations on Experimental Aortic Anastomosis.

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(Introduced by J. C. Hinsey.)

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The following report is based on observations made upon 3 dogs that were subjected to end-to-end anastomosis of the thoracic aorta at the age of 6 weeks and then studied approximately one year after operation.

Method of Study. Six young dogs, male and female litter mates, were subjected to division of the aorta and end-to-end anastomosis at the site of division at ages between 6 and 8 weeks. A seventh male dog was operated on in a similar manner at the age of 3 weeks. The operations were performed under intravenous nembutal anesthesia and endotracheal insufflation. The anastomosis was accomplished with a single continuous everting mattress suture of No. 00000 arterial silk attached to an atraumatic needle.

Four of these dogs died 22 to 42 days after operation from intestinal infestation. Autopsy in each case revealed the

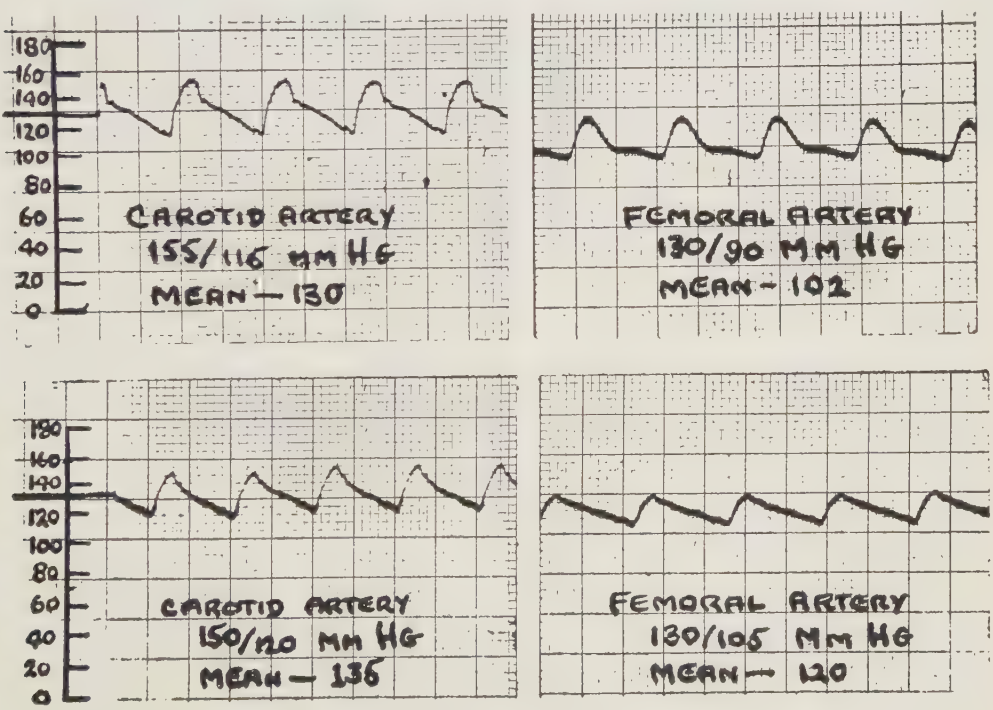

FIG. 1.

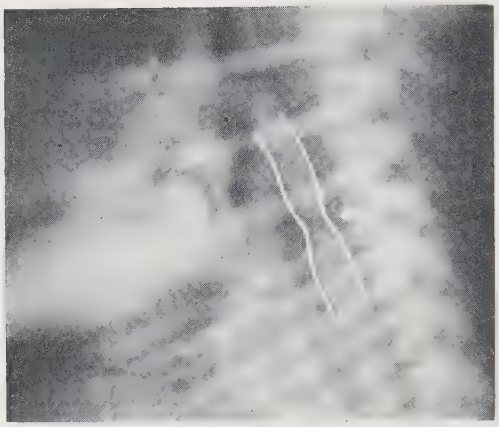

FIG. 2.
Dog 795. Angiocardiogram obtained with 16 cc of neo-iopax. (Retouched).

Femoral pulses were palpable in all 3 animals and blood pressure recordings were obtained with a Sanborn electromanometer in the carotid and femoral arteries of 2 of the dogs. (Fig. 1 and Table I)

Eleven days after angiocardiography, the male dog was subjected to thoracotomy and the site of anastomosis removed. The specimen was submitted to the Department of Surgical Pathology where sections were made longitudinally through the site of anastomosis for microscopic study.

Analysis. Angiocardiographic studies demonstrated in each animal a definite narrowing at the site of anastomosis. (Fig. 2, 3, 4) In one there appeared to be a slight dilation of the aortic arch (Fig. 3); however, there was

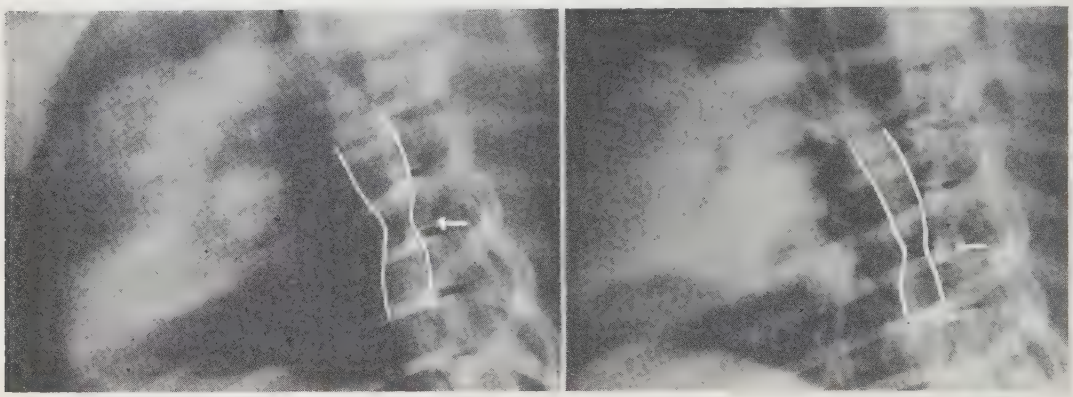

FIG. 3 (left). Dog 797. Angiocardiogram, using 10 cc of 70% diodrast, shows narrowing of thoracic aorta at site of anastomosis. There appears to be some dilatation of the arch of the aorta. (Retouched).
FIG. 4 (right). Dog 798. Angiocardiogram showing slight constriction of thoracic aorta. 14 cc of neo-iopax was used for contrast substance. (Retouched).

suture line to be intact and without evidence of mural thrombosis. The 3 remaining dogs, one male and 2 females, developed normally and at the time of these investigations weighed 28, 34, 34½ lb respectively.

Approximately one year after operation, angiocardiographic studies were made. Under intravenous sodium pentobarbital anesthesia, the left external jugular vein was cannulated. Fourteen to 16 cc of neo-iopax was given rapidly through the cannula in 2 cases, and 10 cc of diodrast in the third. Roentgenograms were obtained at 0.5 sec. intervals throughout the cardiac cycle until the thoracic aorta had been visualized.

no evidence of aneurysmal dilation either above or below the narrow area. There was no suggestion of compensatory collateral circulation in any case.

In the dog subjected to thoracotomy, the area of constriction was readily visualized (Fig. 4). The diameter of the aorta was approximately the same above and below the site of stenosis. It also appeared that the lumen of the anastomotic site was larger than the lumen of the aorta had been when the dogs were 6 weeks of age. The silk suture was still present. (Fig. 5)

Microscopic examination of the resected segment of aorta revealed an intimal prolifera-

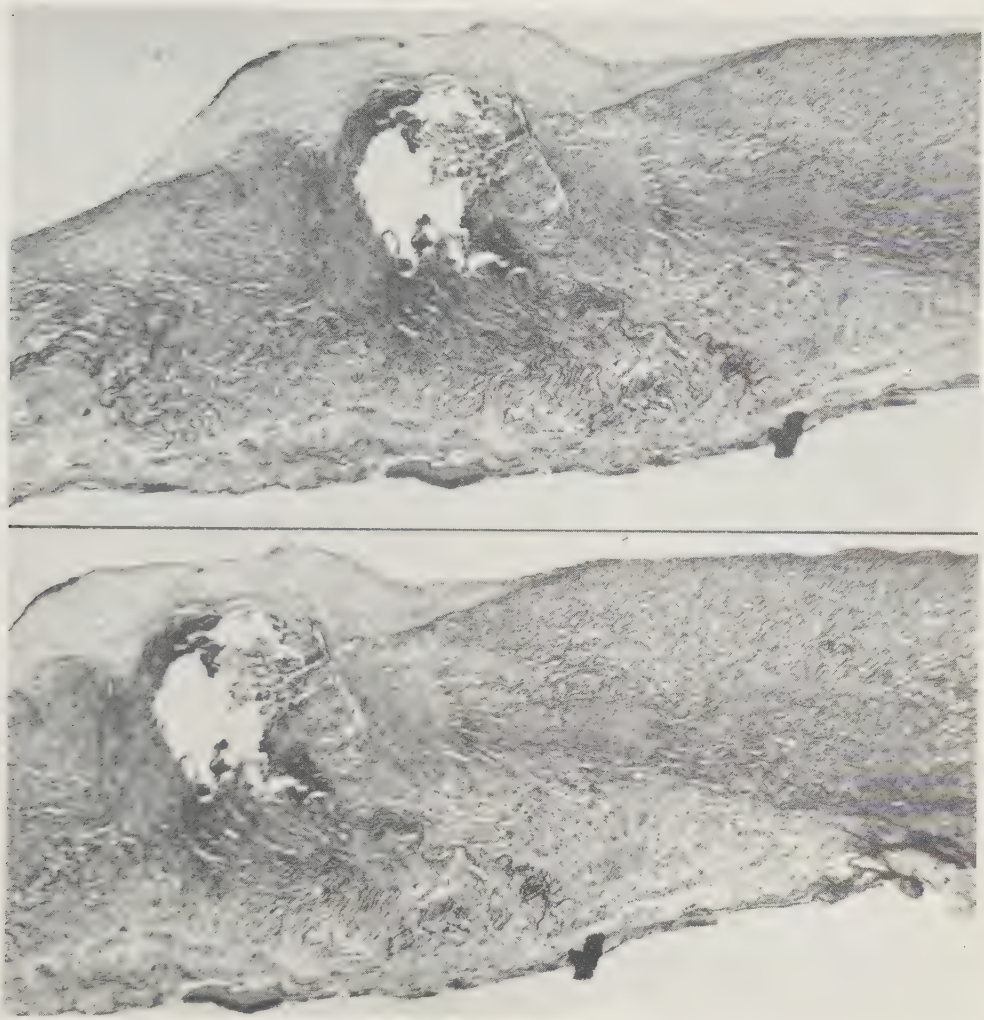

FIG. 5.

Photomicrograph of segment of aorta removed from dog 795. Longitudinal section through the site of anastomosis. In the upper photograph the site of suture is shown in the center of the photograph, the intimal surface being on the superior surface of the section. In the lower photograph the section has been moved to the left in order to show the adjacent aortic wall.

tion at the suture line but atheromatous changes were absent. (Fig. 5) Foreign body reaction was apparent immediately adjacent to the silk suture and there was scarring at the junction. The aortic wall to either side, however, was well preserved. Elastic tissue stains showed good preservation of the media.

Discussion. Interest in surgical intervention of coarctation of the aorta has been active since the report by Crafoord and Nylin[1] of successful excision of the involved segment of aorta and anastomosis. Because the average age at death of 74% of the people afflicted with this congenital lesion has been shown to be 30 years,[2] it is felt that operation should be performed before maturity. In addition, atheromatous changes in the aorta occur early in association with this malformation and accordingly it is technically advantageous to undertake operation at a relatively early age before such changes take place.

[1] Crafoord, C., and Nylin, G., *J. Thoracic Surg.*, 1945, **14**, 347.

[2] Gross, R. E., *J.A.M.A.*, 1949, **139**, 285.

The first case reported by Gross was a 5-year-old boy[3] and, in discussing his experience with 60 cases recently, whose ages ranged from 5 to 30 years, he stated that operation should be avoided before 6 to 8 years of age.[2] Gross indicated that the risks in babies and very young children are high[3] because collateral channels are not well developed and because the aorta is too small for the anastomosis to be accomplished with facility. He stated further than "an aortic lumen may be established at the anastomosis which is satisfactory for a child of a few years of age, but it will probably be insufficient in size when the person grows to maturity."

The experiments reported in this paper appear to have some bearing on the latter two points. There may be some doubt as to the necessity of delaying operation until a child is 6 to 8 years old to assure that the aorta will be large enough to suture without excessive risk. The dogs in this experiment were under 5 lb in weight and less than 8 weeks old at the time of operation, and yet the technical aspects of the anastomoses were accomplished without mishap. The 4 fatalities in the postoperative period were unrelated to the operative procedures.

On the other hand, it would appear that the diameter of the lumen at the site of anastomosis in these dogs did not keep pace in growth with the rest of the aorta. This would support the view that surgical intervention for coarctation should be deferred until the lumen of the aorta is large enough in diameter to insure adequate size as the individual grows to maturity. To be sure, a single continuous suture was used for the anastomosis which might have some bearing on the formation of the constriction.

Summary. Seven young dogs between the ages of three and eight weeks were subjected to end-to-end anastomosis of the thoracic aorta. Three of these dogs survived and have been studied approximately one year after operation to determine the status of the site of anastomosis.

Narrowing of the aortic lumen at the suture line was demonstrated in each animal by angiocardiographic methods and also in one at thoracotomy.

[3] Gross, R. E., *Surgery*, 1945, **13**, 673.

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17276. Isolation of an Anti-thyroid Compound from Rape Seed (*Brassica Napus*).

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In a study of the toxic reactions produced in rats by phenylthiourea and α-naphthylthiourea (ANTU), it was found that rats which had been pretreated with anti-thyroid compounds or fed on certain goitrogenic diets developed a marked resistance to these toxic compounds.[1] This effect has been used as an assay method in the extraction of a goitrogenic compound from rape seed.

Materials and methods. Adult Sprague-Dawley rats of either sex were used in assaying the rape seed fractions. Crude fractions were tested by mixing them with the normal diet of fox chow and feeding for 4 days, following which the rats were injected intraperitoneally with 30 mg/kg of phenylthiourea. More refined fractions were dissolved in water and given in a single subcutaneous injection, followed 24 hours later by 20 mg/kg of phenylthiourea intraperitoneally. Survival of the rats for more than 48 hours after the toxic dose was used as the criterion of activity in the extracts.

The normal LD 50 of phenylthiourea for

[1] Carroll, K. K., and Noble, R. L., *Fed. Proc.*, 1949, **8**, 22.

adult rats was found to be 3 mg/kg but this increased to more than 30 mg/kg in rats which had been fed for 4 days on a diet of fox chow to which 4% of ground rape seed had been added.

Experimental. The following is a detailed account of the extraction of 4 kg of a typical batch of rape seed (variety—Dwarf Essex).

The seed was first ground in a coffee mill and then stirred with 3 volumes of ether. The ether-soluble oil was inactive. The insoluble material (3 kg) was filtered and washed with ether, dried, and then allowed to stand over night in 5 volumes of water. This suspension was filtered with suction through coarse paper and the extraction was repeated with 4 volumes of water. The combined aqueous solution contained most of the active component.

This solution was extracted continuously with ether for 14 days. The ether-soluble fraction consisted of 28 g of yellow oil which was highly active. This oil was shaken with 10 volumes of water which dissolved 75% of the total. The remaining material appeared to be less soluble in water. The yellow solution was filtered and the filtrate (300 cc) treated with 200 cc of 10% silver nitrate solution. The sticky white precipitate was separated by centrifugation, washed twice with water, once each with alcohol, acetone and ether, centrifuging after each washing. The insoluble silver salt appeared to be unstable, turning yellow and eventually black if allowed to remain in contact with the silver nitrate solution. Its stickiness disappeared during the water wash, and the resulting brittle lumps were broken up with a spatula. The dried product was a light-gray amorphous solid insoluble in the common solvents. In this state it could be kept at room temperature for some weeks without appreciable deterioration. Analysis of one sample of this crude material gave the following results.

Calculated for C_5H_6ONSAg:	C–25.4	H–2.56	N–5.94	Ag–45.7
Found	C–22.5	H–2.40	N–6.21	Ag–47.5

The solid (18 g) was suspended in 3% sodium carbonate solution and decomposed with hydrogen sulfide. The precipitate of silver sulfide was separated by centrifugation, washed once with water and the combined aqueous solution was extracted with ether. The ether solution was dried with sodium sulfate and the ether removed *in vacuo,* leaving 5.5 g of a colorless oil which crystallized with difficulty. It could be distilled ($B.P._{0.25}$-160-185°C bath temperature) or purified by crystallization from benzene-ligroin 3:1 (8 cc per g). The recrystallized material melted at 50-51°C and gave the following analysis.

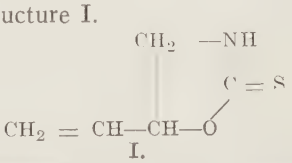
Calculated for C_5H_7ONS:	C–46.5	H–5.47	N–10.8	S–24.8
Found:	C–45.4	H–5.13	N–11.0	S–25.2

The compound gave a blue color with Grote's reagent,[2] which changed to green after 20 minutes and gradually faded.

Its anti-thyroid activity was tested by feeding it to immature rats (0.5% in powdered fox chow) for a period of 11 days. The resultant thyroid hyperplasia was slightly less than that produced when thiourea was fed at a 0.5% level for the same period of time.

Discussion. A variety of mustard oils have previously been isolated[3-5] from plants of the Cruciferae family to which *Brassica Napus* belongs, in some cases by the formation and decomposition of a silver salt.[5] However, thiourea derivatives have also been found to form insoluble silver salts,[6] and the properties of the present compound more closely resemble those of thioureas than of mustard oils.

Using the technic of inhibition of uptake of radioiodine by the thyroid as a method of assay, Astwood, Greer and Ettlinger[7] have isolated what appears to be the same compound from rape seed, and have shown that it has the structure I.



I.

2 Williams, R. H., Jandorf, B. J., and Kay, G. A., *J. Lab. and Clin. Med.,* 1944, **29**, 329.

3 Gadamer, J., *Ber. Deut. Chem. Ges.,* 1897, **30**, 2322.

4 Schneider, W., *Chem. Abst.,* 1910, **4**, 3064.

5 Schmid, H., and Karrer, P., *Helv. Chim. Acta,* 1948, **31**, 1017.

6 Gadamer, J., *Arch. Pharm.,* 1895, **233**, 646.

7 Astwood, E. B., Greer, M. A., and Ettlinger, M. G., *Science,* 1949, **109**, 631.

Their experiments indicated that the anti-thyroid activity of this compound in humans is comparable to that of 6-n-propylthiouracil.

Summary. A new method for the detection of compounds which have anti-thyroid activity is described. This method has been used to isolate a crystalline anti-thyroid compound from rape seed having the empirical formula C_5H_7ONS.

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17277. Effect of Amino Acids and Sodium Bicarbonate on the Level of Glutamine in Blood. V.

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In a previous publication[1] we reported on the depressing effect of glucose and insulin administration on the level of glutamine and amino acids in the blood plasma and some of the possible mechanisms involved in producing this effect were discussed. In a subsequent publication[2] it was shown that this was not due to a mere diffusion of amino acids and glutamine into the tissues as had been suggested by Hamilton.[3]

In this paper we wish to report the effect of the intravenous administration of some amino acids, sodium bicarbonate and physiological saline solution on the level of glutamine in the blood plasma of rabbits.

Procedure and methods. Male rabbits, 18 hours post absorptive, were used for these experiments. Six animals were used for each substance which was tested. The amino acids and sodium bicarbonate were made up as a 10 percent solution in distilled water and 10 ml or the equivalent of 1 g of substance per 3 kg of body weight was administered intravenously. The saline solution was administered in quantities of 10 ml. Since the rabbits were very similar in weight (Table I) the volumes varied by only one or two ml. The rabbits were bled from the marginal vein of the ear just before injection and 30 and 60 minutes after injection. About 12 ml of blood were drawn at each bleeding into tubes containing sodium oxalate to prevent coagulation. The blood was immediately centrifuged for about 10 minutes and the plasma was used for the determination of total amino acids and glutamine. The amino acids were determined colorimetrically by Russell's modification of Frame's method[4] and the glutamine by our method as described in a previous publication.[5] The determinations were carried out in duplicate or triplicate and statistical analysis of the data showed that the variations between duplicate determinations were negligible.

Observations. It will be seen from Table I that the initial level of glutamine in the post-absorptive state bore no relation to the level of the total amino acids during this period.

dl-Alanine. Following the intravenous administration of dl-alanine there was a marked rise in the level of glutamine in the first 30 minutes. This amounted to an average increment of 3.3 mg per 100 ml of blood plasma. Although the level tended to decrease in the following 30 minutes it still was above the initial level by an average of 2.0 mg. Statistical analysis by Fisher's "t"[6] method for a

[1] Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 577.

[2] Harris, M. M., and Harris, R. S., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 471.

[3] Hamilton, P. B., *J. Biol. Chem.*, 1945, **158**, 397.

[4] Russell, J. A., *J. Biol. Chem.*, 1944, **153**, 467.

[5] Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 569.

[6] Mainland, D., Treatment of Clinical and Laboratory Data, p. 147, Oliver and Boyd, London, 1938.

TABLE I.

Effect of Administration of Various Amino Acids, Saline and Sodium Bicarbonate on Level of Glutamine in Blood Plasma.

Rabbit No.	Wt., kg	Substance administered	Amino acid nitrogen in plasma, mg per 100 ml			Glutamine in plasma, mg per 100 ml		
			Before	30 min.	60 min.	Before	30 min.	60 min.
1	3.1		7.0	14.4	10.2	6.0	8.9	6.8
2	3.4	dl-alanine	5.9	14.8	12.7	7.2	10.4	10.6
3	3.5		6.5	16.8	13.4	7.1	9.4	8.4
4	4.0		8.4	19.0	13.2	8.8	12.7	10.5
5	4.4		6.9	14.5	12.8	7.3	11.1	9.0
6	4.0		6.8	14.7	11.5	7.5	11.2	10.3
Average	3.73		6.9	15.7	12.3	7.3	10.6	9.3
						Fisher's "t"	13.6	6.43
				(8.8)*	(5.4)	P	<0.01	<0.01
7	3.9		5.2	17.4	14.4	6.1	8.3	8.3
8	3.9		7.4	18.2	15.3	7.5	9.3	9.5
9	4.1	glycine	7.7	18.7	15.5	8.1	9.6	9.3
10	3.8		6.9	16.8	15.2	11.4	12.5	10.9
11	3.6		6.6	14.1	10.5	7.4	7.7	8.2
12	4.1		6.6	14.6	11.5	8.0	10.1	8.7
Average	3.9		6.7	16.6	13.7	8.1	9.6	9.2
						Fisher's "t"	4.67	4.34
				(9.9)	(7.0)	P	<0.01	<0.01
13	3.4		7.8	19.5	17.1	7.6	8.1	8.3
14	4.0		6.3	21.2	16.7	7.4	8.1	9.3
15	4.2	β-alanine	6.1	19.5	14.9	5.8	8.5	8.0
16	4.3		7.1	21.1	17.6	9.5	10.6	10.1
17	4.2		6.9	21.7	16.3	8.7	8.5	8.7
18	3.6		5.9	21.2	18.9	8.4	9.2	8.8
Average	3.95		6.7	20.7	16.9	7.9	8.5	8.9
						Fisher's "t"	2.35	3.01
				(14.0)	(10.2)	P	>0.05	<0.05
19	4.4		6.6	6.6	6.4	8.0	7.8	6.5
20	4.2		7.4	6.8	6.6	8.4	7.6	8.1
21	3.5	Sodium bicarbonate	7.2	6.8	6.6	7.0	7.7	8.5
22	4.0		6.9	6.2	—	9.8	8.6	8.7
23	4.4		7.2	7.2	7.1	7.6	7.8	8.0
24	3.8		6.4	7.0	7.3	8.3	7.2	8.8
Average	4.05		6.7	6.8	6.8	8.2	7.8	8.1
						Fisher's "t"	1.71	0.160
				(0.1)	(0.1)	P	>0.1	>0.8
25	3.9		6.4	6.3	6.0	6.6	7.0	6.1
26	4.1		6.7	6.1	6.3	7.7	7.1	7.7
27	4.5	Saline	6.4	6.3	6.3	6.5	6.3	5.6
28	3.9		6.9	6.8	6.9	6.9	7.2	7.4
29	2.4		5.9	5.5	6.1	5.0	5.1	5.5
30	3.5		5.9	6.4	5.7	5.9	6.0	6.0
Average	3.72		6.4	6.2	6.2	6.4	6.4	6.4
				(0)	(0)	Fisher's "t"	.206	.096
						P	>0.8	>0.9

* The figures in parentheses represent the increments, above the initial level, of the average level of amino acid nitrogen per 100 ml of plasma.

small number of experimental animals showed that the increments obtained were highly significant for both periods with $P < 0.01$.

Glycine. The administration of glycine produced an average rise in the level of glutamine of 1.5 mg for the 30 minute period and

1.1 mg for the 60 minute period. The "t" values for both periods were significant with $P < .01$. Only one of the 6 animals, (No. 10) showed a slight drop below the initial level in the 60 minute period.

β-Alanine. Following the administration of β-alanine there was an average rise in the level of glutamine of 0.6 mg in the 30 minute period and 1.0 mg after 60 minutes. The "t" value was not significant for the 30 minute period but was significant for the 60 minute period. The value of P was greater than 0.05 for the former and less than 0.05 for the latter period.

Sodium Bicarbonate and Saline. There was a slight drop in the average level of glutamine following the administration of sodium bicarbonate and no change following saline. The "t" values, however, were not significant for any of the periods.

Total Amino Acids Nitrogen. The average values of the total amino acid nitrogen in the different groups were very similar in the initial period before injection. Following the injection of saline and sodium bicarbonate the average values remained practically unchanged in the 30 and 60 minute periods. The administration of the various amino acids resulted in a rise in the level of the total amino acid nitrogen in the plasma which remained elevated with a tendency to fall in the second 30 minute period. The smallest rise occurred with dl-alanine and the highest rise occurred with β-alanine.

Statistical Analysis. The data were analyzed by Fisher's "t" method and the P values for significance were determined from Fisher's table. Any values of P equal to or less than 0.05 were considered significant.

Owing to the fact that the initial average level of glutamine in the plasma was not the same for the various groups, the data were analyzed by the method of variance and covariance by which statistically "adjusted means"[7,8] are obtained.

TABLE II.

Changes in the Level of Glutamine in Blood Plasma Resulting from the Administration of Various Substances Compared with Statistically Adjusted Results by the Method of Variance and Covariance.

Substance administered	Obtained average values of glutamine in plasma, mg per 100 ml					Statistically adjusted mean values of glutamine in plasma, mg per 100 ml				
	Before	30 min.	60 min.	Increment 30 min.	Increment 60 min.	Before*	30 min.	60 min.	Increment 30 min.	Increment 60 min.
dl-alanine	7.3	10.6	9.3	3.3	2.0	7.58	10.81	9.46	3.23	1.88
Glycine	8.1	9.6	9.2	1.5	1.1	7.58	9.20	8.90	1.62	1.32
β-alanine	7.9	8.5	8.9	0.6	1.0	7.58	8.27	8.71	0.69	1.13
Sod. bicarb.	8.2	7.8	8.1	—0.4	—0.1	7.58	7.33	7.74	—0.25	0.16
Saline	6.4	6.4	6.4	0.0	0.0	7.58	7.30	7.09	—0.28	—0.49
Average	7.58	8.58	8.38	1.0	0.8		8.58	8.38	1.0	0.82
F	1.8	12.1	7.5				29.0	8.6		
P	>0.05	<0.01	<0.01				<0.01	<0.01		

* In order to equate for initial level, it is assumed that all of the individual animals showed a "before" average of 7.58 equal to the grand average of all the animals in the "before" determination.

[7] Rider, P. R., Introduction to Modern Statistical Methods, Analysis of Covariance, p. 150, John Wiley and Sons, Inc., New York, 1939.

[8] Snedecor, G. W., Statistical Methods, p. 116, 215, 318, Iowa State College Press, Ames, Iowa, 1946.

The results of both methods of analysis (unadjusted averages and "adjusted means") were essentially similar. (Table II)

It will be noted that the most marked effect was obtained from the administration of dl-alanine and lesser effects were produced by glycine and β-alanine in this order. Since the average values for the initial level of glutamine of the groups of animals used for the administration of sodium bicarbonate and saline, which may be considered as the controls, consisted of both a high and low average value, this also acted as a control for the effect of the variation in the initial level of glutamine.

Discussion. It will be noted that the increment in the level of the amino acid nitrogen after 30 and 60 minutes remained much higher following the administration of β-alanine than that of dl-alanine. This probably indicates that the latter entered the metabolic processes of the tissues more rapidly and is in keeping with the more marked effect on the level of glutamine produced by it.

Christensen and his coworkers[9] have reported that the oral administration to guinea pigs of various α-amino acids, except glutamic acid, will produce a rise in glycine in the plasma with a decrease in the ratio of the concentration of glycine in the liver and muscles to that in the plasma. This was interpreted by them as due to competitive inhibition between some of the amino acids for the means by which the cells concentrate amino acids. However, from a recalculation of their data it is apparent that although the distribution ratio for glycine fell the actual concentration of glycine in the liver cells rose above the fasting level in some cases. It would seem that the rise in the level of glycine in the plasma could be interpreted as resulting from a marked production or accumulation of glycine accompanied by an increased burden placed upon the concentrating power of the liver cells. The authors give no data regarding glutamine except that following the administration of glutamate which resulted in an increase in glutamine production.

If the claims of Christensen and his coworkers are correct, it is possible that the rise in the level of glutamine in the plasma may be due to a displacement of glutamine from the tissues by dl-alanine or glycine. We are inclined to believe, however, that this is probably due to an increased production of glutamine. l-Alanine is known to play an important role in the transamination of glutamic acid to l-alanine (glutamic acid + pyruvic acid ⇔ alanine + ketoglutaric acid). An increase in the supply of alanine would tend to reverse this reaction and thus hinder transamination and favor amidation. Braunstein[10] has indicated that there is an interrelation between transamination and amidation and where transamination is decreased amidation or glutamine formation is increased and vice versa. Since glycine, as far as is known, does not enter into transamination mechanisms the process whereby it produces a rise in the level of glutamine requires further elucidation. It is possible that it may result from the amidation of free ammonia liberated by the amino oxidase of glycine. Various investigators[10,11] have indicated that amidation is probably an important means whereby ammonia is rapidly fixed by the formation of glutamine in the animal organism.

In connection with our observations it may be of interest to call attention to the recent findings of Awapara and his coworkers[12] that the dicarboxylic amino acids are decreased and alanine is increased in the liver of adrenalectomized rats given 17 hydroxydehydrocorticosterone (Compound E of Kendall).

Summary. Studies were carried out regarding the effect of the intravenous administration of dl-alanine, glycine, β-alanine, sodium bicarbonate and saline on the level of glutamine in the blood of rabbits. dl-Alanine was found to produce a marked rise in the level of glutamine. Glycine produced a smaller and β-alanine the smallest rise. Sodium bicar-

9 Christensen, H. N., Streicher, J. A., and Elbinger, R. L., *J. Biol. Chem.*, 1948, **172**, 515.

10 Braunstein, A. E., Advances in Protein Chemistry, Vol. 3, p. 1, Academic Press, Inc., New York, 1947.

11 Krebs, H. A., *Biochem. J.*, 1935, **29**, 1951.

12 Awapara, J., Marvin, H. N., and Wells, B. B., *Endoc.*, 1949, **44**, 378.

bonate and saline produced no rise in the average level of glutamine in the group of animals.

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17278. Effect of Administration of Sodium Benzoate on the Level of Glutamine in Blood. VI.

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In previous publications we reported on the effect of the administration of insulin, glucose,[1] amino acids and sodium bicarbonate[2] on the level of glutamine in the blood. It was suggested that the effect of insulin in lowering the level of glutamine in the blood might be due, in part, to the depression of oxidative deamination of amino acids by insulin. Since it has been reported that benzoic acid depresses the oxidation of d-amino acids[3] and other intermediary metabolites[4,5] the effect of its administration on the level of glutamine was investigated.

Procedure and methods. Male rabbits in the post absorptive state were used for these studies. The effect of the administration of sodium benzoate was studied also in a normal human female subject. The benzoate was administered either intravenously or orally as indicated in the table. Blood was collected in tubes containing sodium oxalate and the plasma separated by centrifugation. Glutamine was determined by the method described in a previous publication.[6] The total amino acids were determined by the method of Simonelli.[7] The blood sugar (total reducing substance) was determined by the Folin-Wu method[8] which includes other non-glucose reducing substances. In some cases the blood was allowed to glycolize at 37°C overnight in order to determine the change in non-glucose reducing substances.

Observations. Doses of 0.25 g, 0.5 g, and 0.75 g of benzoic acid neutralized with sodium hydroxide and injected intravenously produced a fall in the level of glutamine in the plasma both in the 30 min. and 60 min. period after injection. The extent of the fall varied in different animals and was not related to the size of the dose of benzoic acid. Statistical analysis of the fall showed that the drop was statistically significant for both periods (see table).

The level of the total amino acid nitrogen in the blood plasma tended to fall in all animals; however, this was negligible in some animals. The extent of the change in the level of glutamine was not parallel with the changes in total amino acid nitrogen. The blood sugar (total reducing substances) also rose in all the animals which received benzoic acid intravenously. The extent of the rise also was variable and two of the animals which showed the most marked rise (Rabbits nos. 1 and 3) in the "blood sugar" received the smallest dose of benzoic acid. By incubating the blood at 37°C overnight it was found

[1] Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 577.

[2] Harris, M. M., and Harris, R. S., PROC. SOC. EXP. BIOL. AND MED., preceding paper.

[3] Klein, J. R., and Kamin, H., *J. Biol. Chem.*, 1941, **138**, 507.

[4] Griffith, W. H., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 279.

[5] Jowett, M., and Quastel, J. H., *Biochem. J.*, 1935, **29**, 2143 and 2159; Quastel, J. H., and Wheatley, A. H. M., *ibid.*, 1935, **29**, 2773.

[6] Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 569.

[7] Simonelli, U., Clinical Colorimetry with the Pulfrich Photometer, by W. Krebs, p. 24, Carl Zeiss, Jena.

[8] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

TABLE I.
Effect of Administration of Sodium Benzoate on the Level of Glutamine in the Blood.

Rabbit No.	Wt., kg	Substance administered*	Time, min.	Glutamine in plasma, mg per 100 ml	Amino acid nitrogen in plasma, mg per 100 ml	Blood sugar, mg per 100 ml
1	2.5	0.25 g benzoic acid in 8 ml water	0	8.0	9.4	132
			30	6.3	8.7	155
			60	6.8	9.1	220
2	2.6	0.25 g benzoic acid in 8 ml water	0	7.9	10.0	118
			30	5.9	9.7	124
			60	6.0	10.1	152
3	2.9	0.25 g benzoic acid in 5 ml water	0	8.6	9.9	126
			30	6.5	9.8	218
			60	6.6	9.2	260
4	2.9	0.5 g benzoic acid in 10 ml water	0	7.5	10.5	118
			30	6.5	9.8	148
			60	7.5	10.0	172
5	2.9	0.75 g benzoic acid in 10 ml water	0	8.0	8.0	113
			30	7.3	7.3	122
			60	6.9	6.9	119
6	2.9	0.75 g benzoic acid in 10 ml water	0	8.3	7.8	122
			30	7.3	7.3	134
			60	6.9	7.3	163
7	4.2	2.0 g in gelatin capsules + 15 ml water (orally)	0	8.1	7.2	—
			75	5.7	7.1	95
			175	6.1	7.4	96
Human subject	59.1	5.9 g sod. benzoate in capsules + 200 ml water	0	6.6	9.1	89
			45	7.0	8.5	77
			85	6.0	8.4	84
			135	4.8	8.7	92
8		No treatment	0	8.3	9.0	
			30	8.4	9.0	
			60	8.1	9.4	
		Saline controls, avg. for 6 animals see previous paper	0	6.4	6.3	101
			30	6.5	6.2	105
			60	6.5	6.2	109

* Sodium benzoate was administered intravenously except where otherwise indicated.

Statistical analysis of the effect of the intravenous administration of sodium benzoate gave a Fisher "t" value of 6.64 and 6.30, with $P < 0.01$ for the changes in the level of glutamine for the 30 and 60 min. periods respectively, which is highly significant. The corresponding changes in the level of amino acid nitrogen gave a Fisher "t" value of 2.03 with $P > 0.05$ and 1.81 with $P > 0.1$ which were not statistically significant.

in some of the experiments that the rise was due in part to an increase in a reducing substance which did not undergo glycolysis and was undoubtedly due to glycuronic acid.

Sodium benzoate was administered orally in capsules to one rabbit and one human female subject in doses of 2.0 g and 5.9 g respectively. The level of glutamine dropped markedly in both cases. However, the level of amino acid nitrogen remained practically unchanged in the rabbit. In the human subject the glutamine level dropped 27% and the total amino acid nitrogen only about 4% at the end of 135 min. after the administration of sodium benzoate. The "blood glucose" showed very little change in both cases and in fact was slightly lower in the human subject after 45 minutes.

Discussion. It is claimed that benzoic acid is detoxified by forming a glycuronide in the

rabbit and hippuric acid in man. If this is so, it would mean that the level of glutamine is depressed irrespective of the mode of detoxification used by the animal organism. It is interesting to note the variable effect of the administration of benzoic acid on the level of the total amino acids and the total reducing substances in the blood. Although glycine was withdrawn from the metabolic mixture as hippuric acid in the experiment on the human subject (several grams of crystalline hippuric acid were isolated from her urine), still the level of the total amino acids in the blood was lowered to no greater extent than in some of the rabbits. These variable effects in the different animals suggest that variable metabolic changes, besides that involved in the formation of benzoyl glycuronide, probably occur in different rabbits. The fall in the level of glutamine may be due, in part, to the inhibition of the oxidative deamination of amino acids beside the inhibition of other oxidative processes.[4,5] This would reduce the supply of nitrogen for amidation. However, where there was a fall in the level of the total amino acids in the blood it is possible that certain amino acids were withdrawn from the metabolic mixture thus reducing the supply of nitrogen for amidation. The inhibition of deamination alone should tend to raise the level of the amino acids in the blood. Since in some cases the level of glutamine was depressed without any significant change in the level of the total amino acids (Rabbits nos. 2, 3 and 7) it is possible that the process of amidation was directly depressed.

Christensen and his co-workers[9] claim that special mechanisms are present in cells for concentrating amino acids and that in the presence of high concentrations of amino acids there is competition between the various amino acids for these mechanisms. If the depression in the level of glutamine in the plasma were due to a shift of glutamine into tissue cells due to the removal of glycine by conjugation with benzoic acid then one should obtain a significant drop in the level of the total amino acid nitrogen in the plasma. This did not occur in a number of experiments. Furthermore, the statistical analysis indicated that the changes in the level of amino acid nitrogen were not significant while those for glutamine were highly significant (see additional discussion in previous paper and table). This problem requires further study.

It is of interest to note that the smaller doses of sodium benzoate (Rabbits nos. 1 and 3) produced the most pronounced rise in blood sugar (total reducing substances). The reason for this effect is not apparent at the present time.

Summary and conclusions. 1) Sodium benzoate administered intravenously and orally to rabbits and one human subject was found to depress the level of glutamine in the blood plasma.

2) The intravenous administration of sodium benzoate produced a rise in the total reducing substances in the blood which was highest with the smallest doses of benzoate.

3) The changes in the glutamine level were not related to either the change of the total amino acid level or the level of total reducing substances in the blood.

4) It is suggested that amidation may be either directly or indirectly depressed by the administration of sodium benzoate.

[9] Christensen, H. N., Streicher, J. A., and Elbinger, R. L., *J. Biol. Chem.*, 1948, **172**, 515.

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17279. Aureomycin in Experimental Polyarthritis with Preliminary Trials in Clinical Arthritis.*†

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A polyarthritis of rats can readily be reproduced by intraperitoneal or intravenous injection of broth cultures of the L_4 strain of pleuropneumonia-like organism. This experimental polyarthritis has been used as a means of making chemotherapeutic trials.[1–3] In man pleuropneumonia-like organisms have repeatedly been isolated from the genitourinary tract and "may be related etiologically to an acute infectious type of arthritis and to Reiter's syndrome."[4] Dienes has isolated L type cultures from many Gram negative bacilli and several large Gram positive bacilli,[5] although thus far no one has reported isolation of such forms from streptococci. Unfortunately these microorganisms are species specific as far as their pathogenicity is concerned so that human strains do not produce infections in animals. While the polyarthritis of rats is not the same disease as rheumatoid arthritis in man, the course of both is favorably altered by the use of gold salts. In the rat the arthritis may be prevented by the intramuscular injection of gold at the time of infection, or after the arthritis is developed, treatment with gold will promote healing more rapidly than in untreated controls.[1] Our effort in the chemotherapy of this rat arthritis has been directed toward finding agents which might be as effective as gold, but less toxic, for possible trials in rheumatoid arthritis of man. The new antibiotic, aureomycin, has proved to be successful in this experimental polyarthritis.

Methods. Aureomycin was given to the infected rats by stomach tube, by subcutaneous injection, or by mixing with the diet. The effect of aureomycin both as a preventive and a curative agent was evaluated. Scoring was designed to include the per cent incidence of arthritis in each group of white rats tested, and the per cent survival. The extent of the joint involvement was evaluated according to a modification of the arthrogram of Sabin and Warren[6] which assigns a numerical value of 4 to each front leg and 5 to each hind leg, giving a total of 18 points per animal assuming maximal joint involvement of all joints. The average arthrogram scores were determined by adding the total scores for each group and dividing by the number of animals in the group irrespective of per cent incidence of arthritis.

The microbe was cultured according to a method described by Tripi and Kuzell[1] in a broth culture which is a buffered yeast-extract tryptose base enriched with 20% filtered horse serum. Table I shows the protective effect of aureomycin given coincident with the intraperitoneal injection of 2 cc of a broth culture of the microorganism or shortly thereafter.

Results. When 0.1% or 0.3% aureomycin was mixed with the ground Purina dog chow complete protection of all animals occurred (Table I). When aureomycin was adminis-

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† The aureomycin was supplied by Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y.

1 Tripi, H. B., and Kuzell, W. C., *Stanford Med. Bull.*, 1947, **5**, 98.

2 Findlay, G. M., Mackenzie, R. D., and MacCallum, F. O., *Brit. J. Exp. Path.*, 1940, **21**, 13.

3 Sabin, A. B., and Warren, J., *Science*, 1940, **92**, 535.

4 Dienes, L., Ropes, M. W., Smith, W. E., Madoff, S., and Bauer, W., *New England J. Med.*, 1948, **238**, 509 and 563.

5 Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 30.

6 Tripi, H. B., Gardner, G. M., and Kuzell, W. C., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 45.

TABLE I.
Preventive Effect of Aureomycin in Polyarthritis of Rats.

% aureomycin in diet	No. of animals*	Procedure	Incidence of arthritis, %	Avg arthrogram score	Survival, %
0.3	20	Aureomycin added on day of infection and continued for 7 days	0	0	100
0.1	20	Aureomycin added on day following infection and continued for 11 days	0	0	100
0.05	26	Aureomycin added on day of infection and continued for 14 days	15.0	0.07	100
0	10	Intubation dose 50 mg/kg — Aureomycin given by stomach tube (single daily dose) for 2 days following infection	20.0	0.4	100
0	10	20 mg/kg — Aureomycin given by stomach tube (single daily dose) for 2 days following infection	20.0	0.38	100
0	10	Single dose of aureomycin (100 mg/kg) subcutaneously on day of infection	20.0	0.30	100
0	76	Untreated controls	77.4	2.41	96

* The average weight of the groups at the beginning was 67 to 75 g.

tered by stomach tube to fasting animals in single daily doses of 50 mg per kg for 2 days, there was an incidence of 20% arthritis with an average arthrogram score of 0.4, while the incidence among the controls was 77.4%, and the average arthrogram score was 2.41, the survival rate being 96%. A single dose of aureomycin, 100 mg per kg, given subcutaneously on the day of infection gave an incidence of 20% and an average arthrogram score of 0.3, compared to a much higher value (2.41) for the controls (Table I).

The curative effect was evaluated by giving aureomycin 100 mg per kg subcutaneously on the 7th and 8th days after the infection was begun and at a time when there was a 55% incidence of arthritis. The crude aureomycin being quite acid caused local necrosis, so further injections were not given. In 4 days the incidence of arthritis had fallen from 55 to 5% in the treated group while it had increased from 66 to 73% in the controls (Table II). At the same time the average arthrogram score for the treated animals had decreased in 4 days from 1.6 to 0.15 while in the controls it had increased from 1.3 to 1.35. At the outset there was a less severe degree of arthritis in the controls, the drug being tested against a more severe arthritis, yet the outcome was more favorable.

In vitro, a concentration of 2 μg aureomycin per cc of the broth permitted questionable growth of the microbes in 24 hours and definite growth in 48 hours as estimated by the turbidity of serial dilutions. Using 3 μg of aureomycin per cc of broth, no growth was observed.

Estimations of blood levels‡ of aureomycin in the treated rats, according to the method of Brainerd *et al.*,[7] showed that rats receiving 0.1% aureomycin in the Purina dog chow for 3 days had blood levels of 0.62 μg per cc, while those eating a diet containing 0.075% aureomycin had less than 0.155 μg per cc. Rats receiving 50 mg per kg with a stomach tube once daily for 2 days showed blood levels of 0.62 μg of aureomycin per cc when the sample was taken 2 hours after the adminis-

‡ We are indebted to Miss Mirra Scaparone of the San Francisco Hospital for determination of the aureomycin blood levels.

7 Brainerd, H. D., Bruyn, H. B., Jr., Meiklejohn, G., and Scaparone, M., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 318.

TABLE II.
Curative Effect of Aureomycin in Polyarthritis of Rats.

	No. of animals*	Incidence of arthritis: Pretreatment, %	Incidence of arthritis: 4 days after aureomycin, %	Avg arthrogram score: Pretreatment	Avg arthrogram score: 4 days after aureomycin
100 mg aureomycin per kg subcutaneously on 7th and 8th days after infection	20	55	5	1.6	0.15
Untreated controls	20	66	73	1.3	1.35

* The average body weight in both groups was 75 g.

tration of the drug. The untreated controls showed no measurable level of the antibiotic.

Preliminary clinical trials were made in 4 advanced cases of rheumatoid arthritis which had responded unsatisfactorily to several therapeutic agents, and one early case using 2 g aureomycin daily by mouth for 1 month. Two patients noted an improvement in appetite, and one patient had to discontinue the medication on the third day due to a marked gastrointestinal upset. None of these cases showed any improvement in range of motion of affected joints and 3 of them developed increased pain and swelling while under treatment. One case of Reiter's syndrome responded dramatically, gaining 6 lb during the first week of treatment and losing all joint pain. At the end of one month he showed no more joint swelling. His blood sedimentation rate (Wintrobe Method) decreased from 40 mm to 12 mm per hour in 2 weeks.

Summary. 1. The addition of aureomycin to the diet, subcutaneous administration, and gastric intubation of the antibiotic prevented and cured experimental polyarthritis of rats due to the L_4 strain of pleuropneumonia-like organism.

2. *In vitro* aureomycin prevented growth of the microbe in broth.

3. In preliminary clinical trials, several patients with chronic rheumatoid arthritis who responded unsatisfactorily to several therapeutic agents also failed to respond to aureomycin.

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17280. Effect of Incubation on the Cholesterol Partition in Human Serum.*

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Sperry[1] reported that the incubation of blood serum or plasma from normal human subjects resulted in a decrease in the amount of free cholesterol present without a change in the total cholesterol. It was concluded that esterification of some of the free cholesterol had taken place and that this had come about through the action of an enzyme, as the process was abolished by heating the serum to 55-60° prior to incubation. If this esterification of cholesterol *in vitro* were the result of enzymatic activity, it seemed possible that the enzyme might be absent or present in reduced amounts in the peripheral blood of patients with an abnormally low cholesterol ester fraction in the serum. This would

* This investigation was supported by Research Grant No. H39 from the National Heart Institute, U. S. Public Health Service.

1 Sperry, W. M., *J. Biol. Chem.*, 1935, **111**, 467.

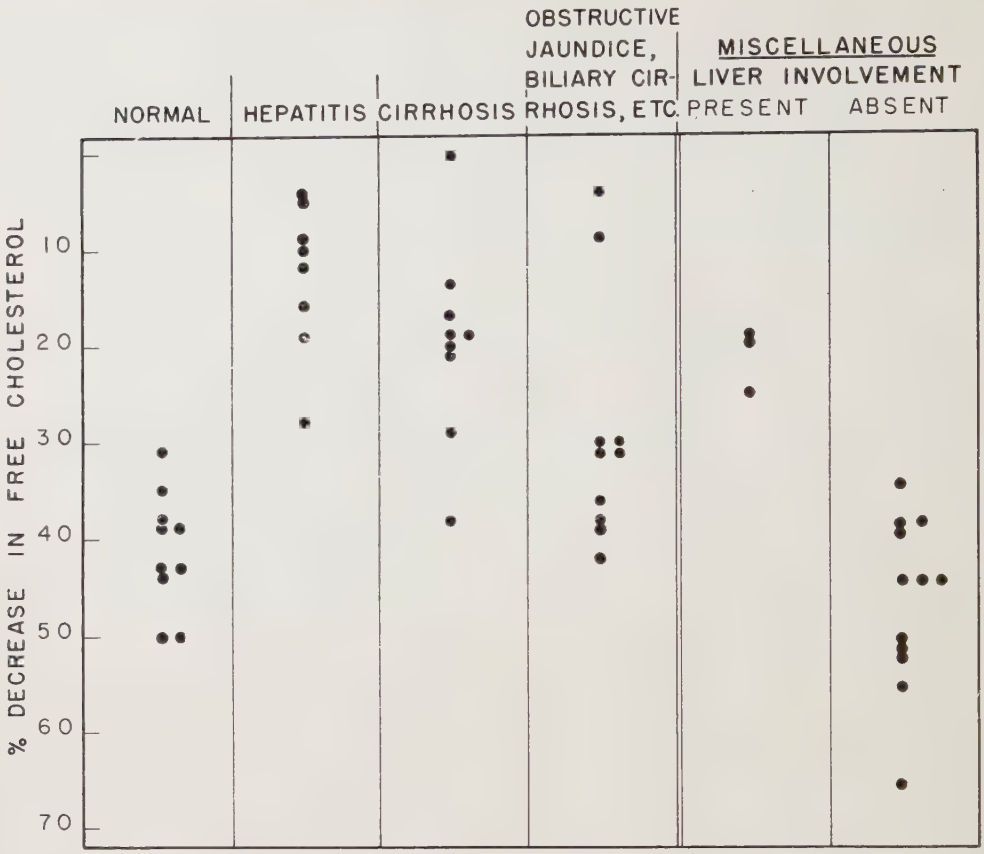

FIG. 1.

The percentage reduction in the amount of free cholesterol in the serum after incubation at 37° for 24 hours. Each dot represents one patient.

occur primarily in liver disease. To test this hypothesis the present investigation was undertaken.

Experimental. The effect of incubation on the serum cholesterol partition was studied in 10 normal individuals, 30 patients with liver disease, and 12 patients with a variety of disease conditions but without liver involvement. The concentration of total and free cholesterol in each sample of serum was determined by the method of Schoenheimer and Sperry[2] before and after incubation at 37° for 24 hours. The difference between the total and free cholesterol was assumed to represent the esterified cholesterol. Blood samples were usually obtained in the fasting state. No preservative was added to the serum before incubation. Hemolyzed samples were discarded as Sperry had found that hemolysis inhibits the reaction, and we had confirmed this observation.

The values obtained for the total cholesterol in each specimen before and after incubation were mostly within the limits of error of the method. In other words, the total cholesterol did not change. The decrease in the amount of free cholesterol with incubation varied from none to 69%. The results are summarized in Fig. 1.

In a number of instances the period of incubation was extended to 48 or 72 hours. This seemed to provide little added information even though a further decrease was observed in the amount of free cholesterol, and consequently the 24-hour period was adopted as standard procedure. In one case the decrease

[2] Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.

TABLE I.
Effect of Heating Serum in Preventing a Decrease in Free Cholesterol with Incubation.

		Serum cholesterol				
			Free		Ester	
		Total mg %	mg %	% decrease	mg %	%
Subject 8	Control	268	75		193	72
	Incubated*	267	76	0	191	72
	" †	268	49	35	219	81
Subject 9	Control	180	47		133	74
	Incubated*	181	47	0	134	75
	" †	181	27	43	154	85

* After heating to 56° for one-half hour.
† Unheated.

in free cholesterol was determined after incubation for 3, 6, 12 and 24 hours. At 3 hours the drop amounted to 13%; at 6 hours it was 18%; at 12 hours, 32% and at 24 hours 42%.

We have confirmed Sperry's observation[1] that heating the serum to 56° before incubation prevents the drop in free cholesterol from taking place, presumably by destroying the enzyme responsible for the reaction. Data on the effect of preliminary heating on the serums of 2 normal subjects are shown in Table I.

Results. The decrease in the free cholesterol in the serums of 10 normal individuals ranged from 31% to 50% following incubation. Accordingly, decreases of more than 30% in 24 hours are considered normal, and a decrease of less than 30% is considered abnormal. This agrees with the results of Sperry[1] who, in 30 samples from 22 healthy young adults, obtained a decrease in free cholesterol of 29 to 84% after incubation for 24 to 72 hours.

Among the 30 patients with liver disease there were 8 with acute hepatitis. In all of these the drop in free cholesterol with incubation was less than normal, suggesting that the amount of enzyme present was decreased in this disorder. The decrease in free cholesterol ranged from 4% to 19% in 7, and was 28% in the remaining case. All of the patients in this group showed a low ester cholesterol (25 to 50%) prior to incubation. The serum bilirubin was elevated in all. The cephalin flocculation test was positive in 4, negative in 4. The alkaline phosphatase exceeded 5 Bodansky units in 5. In 2 patients the serum albumin was less than 4.0 g%. In general, there did not seem to be a close correlation between these other tests and the degree of reduction of free cholesterol on incubation.

In 8 of 9 patients with Laennec's cirrhosis of the liver, the decrease in free cholesterol with incubation was less than the empirical normal, ranging from none to 29%. The proportion of esterified cholesterol before incubation was slightly below normal (60 to 69%) in 7, and low (18%) in one. The serum bilirubin was increased in 7. The cephalin flocculation test was positive and the serum albumin reduced also in 7 cases. The alkaline phosphatase was between 5 and 6 Bodansky units in 3; less than 5 units in 5. The ninth patient in the group was admitted to the hospital because of bleeding esophageal varices, and was considered to have inactive cirrhosis. The serum of this patient showed a normal drop of 38% in free cholesterol after incubation, and all other chemical tests were normal as well.

In another group were 10 patients with obstructive jaundice, biliary cirrhosis, or extensive metastatic carcinoma of the liver. A characteristic of the group was an elevated alkaline phosphatase exceeding 10 Bodansky units. A reduction in free cholesterol following incubation that was interpreted as being within the normal or low normal range occurred in 8 of the 10 cases. The proportion of esterified cholesterol before incubation was less than 70% in 9 of these 10 cases. The

serum bilirubin was elevated in 7, the cephalin flocculation test was positive in one, abnormal A/G ratios were present in 2.

In a final group among the examples of liver disease there were 3 patients with miscellaneous disorders. One patient had disseminated lupus with ascites, a marked reduction in serum albumin, and a positive cephalin flocculation. Another was admitted in severe congestive heart failure with clinical jaundice and was found to have an elevated serum bilirubin, positive cephalin flocculation, and 50% bromsulfalein retention. The third patient showed extreme involvement of the liver by Hodgkin's disease at autopsy. The decrease in free cholesterol following incubation of serum from these 3 patients was interpreted as less than normal in all—19%, 25% and 20%.

As a further control, 12 patients with various diseases but without demonstrable liver involvement were studied. The group included 4 cases of anemia, 2 cases of fever of unknown origin, and single examples of optic neuritis, leukemia, chronic glomerulonephritis, diabetes, ichthyosis, and psoriasis. In all of these patients the reduction of the free cholesterol with incubation was considered normal. The decrease ranged from 35% to 69%. In 8 of these cases the proportion of esterified cholesterol before incubation was above 70%; in 4 it was between 58% and 69%.

Two or 3 determinations of the reduction of free cholesterol have been made at varying intervals on the serums of each of 6 patients. In a diabetic the decrease was constant on 3 occasions within 8 days. In two patients with infectious hepatitis who showed a decreased reduction in the amount of free cholesterol originally, the test returned to within normal limits with clinical improvement; in a third patient with this disease there was a slight progressive decline in the reduction of free cholesterol until the death of the patient. A patient with cirrhosis, who at first had an abnormally slight decrease in the amount of free cholesterol with incubation, later showed a reduction considered within the normal range. This was accompanied by very little change in clinical condition and chemically only by a rise in serum albumin from 2.7 g to 3.3 g. A patient with heart failure and clinical jaundice, who at first had a reduction of 25% in free cholesterol with incubation showed a normal reduction of 46% after clinical improvement and disappearance of the jaundice.

Discussion. The mechanism of the reaction described is not clear. An enzyme appears to be involved in the reduction in the amount of free cholesterol in the serum following incubation. This enzyme effect is decreased when the liver parenchyma is involved as in hepatitis and cirrhosis, and also occasionally in obstructive jaundice. Work is in progress in an attempt to elucidate the mechanism and to establish the clinical significance of the reaction here described.

The decrease in free cholesterol with incubation does not parallel the rise in serum bilirubin. Sperry[3] found that the addition of bile salts to the serum *in vitro* inhibited the reaction, but we have found a normal reduction in free cholesterol in the presence of clinical jaundice and a decreased reduction without an increase in the serum bilirubin. There is certainly no correlation between this test and the cephalin flocculation test, alkaline phosphatase or serum albumin. There seems to be a somewhat better correlation with the proportion of esterified cholesterol in the serum before incubation, but even in this there are striking exceptions.

While the change in free cholesterol has been given in percentages, it should be pointed out that it may eventually prove desirable to express this in milligrams per cent. Decision on this point must await further information from work now in progress on the nature of the enzyme and its action.

Summary. Incubation of normal human serum and that of patients with disease not involving the liver results in a drop in free cholesterol of more than 30% without change in the total cholesterol. The reaction is probably due to an enzyme, as the decrease does not occur when the serum is heated to 56° before incubation.

[3] Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, 1937, **117**, 525.

In this small series, the decrease in free cholesterol with incubation was usually less than normal with disease of the liver parenchyma and occasionally with extra-hepatic obstructive jaundice. The decrease in free cholesterol apparently did not parallel changes in serum bilirubin, alkaline phosphatase, serum albumin, or the cephalin flocculation test. There appeared to be some correlation with the percentage of cholesterol esters in the control serum.

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17281. Effect of Antihistamine on the Localization of Trypan Blue in Xylene Treated Areas of Skin.

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Some data and experimental observations have suggested that histamine may play a fundamental role in the development of local areas of inflammation.[1-3] In support of this, it has been shown that trypan blue following an intravenous injection localizes and concentrates in areas of skin previously injected with histamine.[4] The effects of antihistamine drugs on capillary permeability have been reviewed recently by Last and Loew.[5] These investigators found that the localization of trypan blue in areas of skin previously injected with different chemical and biological preparations was not modified by the intravenous injection of the antihistamine preparation "Benadryl".[5]

The present experiments were performed to study the effect of two of the more recent antihistamine preparations on the hyperemia and the localization and concentration of trypan blue in areas of skin treated with xylene.

Methods and materials. Fifteen rabbits were used. Their hair was carefully removed 24 to 48 hours preceding the time of the experiment. Two antihistamine preparations were used: Pyrrlidineethyl-phenothiazine hydrochloride (Pyrrolazote*); and, N,N-Dimethylly-N′ (alpha-pyridyl)-N′ (alpha-thenyl)-ethylenediamine hydrochloride (Thenylene†). A solution of each containing 10 mg per cc was made in physiologic sodium chloride. The injections of Pyrrolazote were made intravenously, while Thenylene was injected both intravenously and intraperitoneally. Ten cc of a 0.2% solution of trypan blue was injected intravenously. Xylene was carefully applied with a cotton applicator to local areas of skin at intervals varying from one to 140 minutes during the time that the rabbits were under the influence of the antihistamine preparations and before the dye was injected intravenously. The xylene treated areas were carefully observed during the development of hyperemia and during the time of the localization and concentration of the dye. These observations extended over a period of 2 hours.

In 8 rabbits 0.2 cc of the antihistamine preparations was injected intradermally from immediately to 60 minutes preceding the time of the intravenous injection of trypan blue. An equal volume of both a physiologic sodium chloride solution and distilled water was used as controls.

1 Lewis, Sir Thomas, London, Shaw and Shaw, 1927.

2 Findlay, G. M., *J. Path. and Bact.*, 1928, **31**, 633.

3 Mayer, R. L., *Ann. Allergy*, 1947, **5**, 113.

4 Rigdon, R. H., *J. Lab. and Clin. Med.*, 1942, **27**, 1554.

5 Last, M. R., and Loew, E. R., *J. Pharm. and Exp. Therap.*, 1947, **89**, 81.

* Obtained from the Upjohn Company, Kalamazoo, Mich.

† Obtained from the Abbot Research Laboratories, Chicago, Ill.

Experimental. Four rabbits were given Pyrrolazote intravenously. One received 23.31 mg/kg in 5 injections during a period of 85 minutes; a second, 24.21 mg/kg in 4 injections during a period of 48 minutes; a third, 15.54 mg/kg in 3 injections during a period of 137 minutes and the fourth, 16.5 mg/kg in 2 injections during a period of 16 minutes.

The areas of skin where xylene was applied became hyperemic within a minute and progressively increased in intensity for approximately 5 minutes, after which the reaction remained more or less constant for several hours then regressed. The rate of development and the intensity of the hyperemia that occurred following the application of xylene did not vary from that in rabbits similarly treated, but not given any antihistamine as previously reported.[6] Trypan blue likewise localized and concentrated in these xylene treated areas in a manner similar to that observed in rabbits not given any antihistamine.

All the xylene treated areas of skin were hyperemic. Trypan blue, however, localized and concentrated first in the last 2 areas where xylene was applied the shortest interval before the dye was given.

Three rabbits were given Thenylene both intravenously and intraperitoneally. One was given 28.11 mg/kg in 6 injections over a period of 37 minutes, another 16.64 mg/kg in 4 injections over a period of 35 minutes, and one, 17.57 mg/kg in 4 injections during a period of 40 minutes.

The areas of skin where xylene was applied were identical in these rabbits with those observed in animals given the Pyrrolazote and trypan blue. These 3 rabbits showed toxic symptoms produced by this antihistamine preparation during the time of the experiment.

Trypan blue localized and concentrated in each skin area of the 8 rabbits where both antihistamine drugs were injected intradermally. In 2 rabbits injected with Thenylene there was less dye after 30 minutes in the areas injected 35 minutes before the intravenous injection than in the areas injected 15 minutes and immediately preceding the intravenous injection of the dye. Trypan blue did not concentrate in any of the areas of skin previously injected with physiologic sodium chloride. Twenty minutes following the intravenous injection of trypan blue there was a small area at the site of the injection of the antihistamine preparation that was pale in color and did not stain blue. There was a zone of dye around this anemic area and, peripheral to it, there was a zone of edema which did not stain any deeper with trypan blue than surrounding normal skin. There were no significant changes in the manner of localization of trypan blue in the injected areas of skin after the first 30 minutes of the experiment.

Discussion. In these observations the two antihistamine preparations, Thenylene and Pyrrolazote, in the concentrations used apparently do not effect the development of hyperemia and the localization and concentration of trypan blue in xylene treated areas of skin. In previous studies it has been emphasized that the localization and concentration of trypan blue in areas of inflammation are not determined by the presence of hyperemia.[6] In support of this observation, Last and Loew have found that acetyl-B-methyl-choline (Mecholyl), although producing vasodilation, did not cause a trypan blue reaction.[5] Can it be that a local area of skin treated with xylene or injected with horse serum stains blue primarily as a result of a variation in the absorptive ability of the tissue cells, and not because there is only a change in the permeability of the capillaries? The vital staining of cells has been regarded by some as due to absorption of dye molecules by cell granules.[7]

According to Last and Loew,[5] appropriate concentrations of intradermally injected horse serum, tetracaine, codeine and heparin cause positive trypan blue reactions which are not modified by Benadryl. Arginin, which is a specific antagonist against some of the effects of histamine, does not prevent the production of a trypan blue reaction by either histamine or agents which liberate histamine.[8] The above observations are different from those

[6] Rigdon, R. H., *Arch. Surg.*, 1940, **41**, 101.

[7] Nagao, K., *J. Inf. Dis.*, 1921, **28**, 294.

[8] Rocha e Silva, M., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **73**, 405.

of Mayer who found that "pyribenzamine, a substance exhibiting strong and specific antihistamine properties, exerts a definite activity in experimental dermatitis".[3]

The localization and concentration of trypan blue following an intravenous injection, in areas of skin injected intradermally with antihistamine emphasizes the fact that substances other than histamine may cause a localization of this dye.

Summary The hyperemia that follows the local application of xylene apparently is not modified by the intravenous and intraperitoneal injections of the antihistamine preparations, Pyrrolazote and Thenylene. Likewise, the localization and concentration of trypan blue in the xylene treated areas of skin are not affected by these preparations of antihistamine. Trypan blue also localizes and concentrates in areas of skin injected intradermally with these preparations of antihistamine.

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17282. Studies on Elimination of Penicillin G in Dogs.*

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Rammelkamp and Keefer[1] found that only about 60% of intravenously administered penicillin could be recovered from the urine of man. This finding has been confirmed by later reports, although the recovered amounts vary from 40 to 99%.[2]

This paper deals with the mechanism of the elimination of penicillin in bilaterally nephrectomized dogs.

Methods. Healthy mongrel dogs weighing from about 6 to 12 kg were used. Crystalline penicillin G§ was injected intravenously in all experiments; the blood samples were obtained from another vein and the serum penicillin assayed against Staphylococcus 209 by a serial dilution technic.

Diffusion of Penicillin from Blood to Tissues. Results. A dog which had been bilaterally nephrectomized and the cystic and common bile ducts ligated was given an intravenous injection of 25,000 u/kg of crystalline penicillin G. The penicillin concentration decreased rapidly during the first hour but very slowly thereafter (Fig. 1). It is assumed that this initial decrease was due to

The diffusion of penicillin from blood to tissues

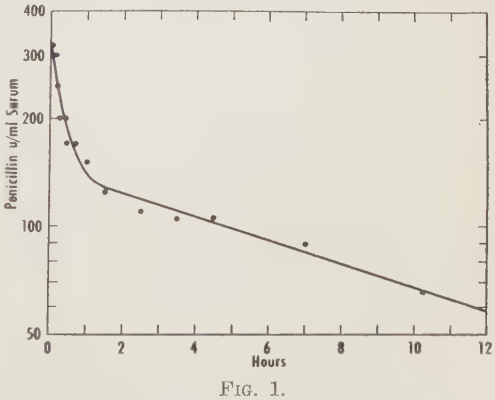

FIG. 1.

The serum penicillin concentration as a function of the time after a single intravenous injection. The inactivation has been partially blocked by bilateral nephrectomy and ligation of the cystic and common bile ducts.

* This investigation was supported, in part, by a research grant from the Antibiotics Study Section of the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and, in part, by the A. B. Kuppenheimer Foundation and the Douglas Smith Foundation of the University of Chicago.

† Douglas Smith Foundation Fellow in Medicine.

‡ Research Fellow, U. S. Public Health Service.

1 Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

2 Herrell, W. E., Penicillin and Other Antibiotic Agents, W. B. Saunders Company, 1945.

§ The crystalline penicillin was supplied by Abbott Laboratories, Commercial Solvents Corporation, Lederle Laboratories, and Schenley Laboratories, Inc.

TABLE I.

Dose units/kg	c ∞ units/ml	c ∞ units/ml dose units/g of dog
85,000	400	4.7
80,000	250	3.1
30,000	130	4.3
25,000	90	3.6
25,000	70	2.8
25,000	80	3.2
25,000	100	4.0
25,000	85	3.4
25,000	100	4.0
25,000	110	4.4
25,000	80	3.2
25,000	100	4.0
25,000	140	5.6
14,000	60	4.3
14,000	60	4.3
7,000	20	2.9
	Average — 3.9 Maximum—5.6 Minimum—2.8	

The serum concentration (c ∞) of penicillin after diffusion equilibrium has been established with the tissues. Values are corrected for any elimination or inactivation of penicillin taking place.

diffusion of penicillin from the blood stream into the tissues.

If there were no further elimination of penicillin from the blood, the concentration would decrease to a constant value which would be reached when diffusion equilibrium was established. This concentration may be found by drawing the straight line representing the inactivation back to intersection with the axis of the ordinate which, in this case, was 143 u/ml of serum.

The equilibrium concentration was determined in a number of experiments and collected in Table I. In the last column of this table are the ratios between c ∞ (serum concentration of penicillin at equilibrium) and the dose of penicillin per gram of dog. In 16 experiments this ratio was found to be larger than unity. This means that the penicillin did not distribute equally throughout the tissues of the dog but was present in a higher concentration in the plasma than in the remainder of the dog. The average ratio was about 4 times as great when diffusion equilibrium was established. This relationship can be expressed as:

Serum penicillin concentration = 4 x amount of penicillin per gram body weight. The formula has been established for the blood concentrations between 20 and 400 u/ml and may not be valid for smaller concentrations.

The Renal and Extra-Renal Elimination. Blood serum concentrations of penicillin were determined at intervals after a single intravenous injection in a number of normal dogs. When allowance was made for the diffusion equilibrium to be established, it was found that the logarithm of the concentration decreased as a linear function of time (First order reaction). This relationship was found to be valid from the initial concentration of about 100 u/ml to 1 u/ml. A curve demonstrating this relationship is shown in Fig. 2.

Bilateral nephrectomy was performed in 3 dogs which were then given a single intravenous injection of penicillin. These dogs were still able to eliminate penicillin from the blood stream, although at a considerably lower rate (Fig. 2). The average rate of elimination was found to be ¼ as rapid in the nephrectomized as in the normal dogs. This indicates that approximately three-fourths of the penicillin was eliminated through the kidney and one-fourth elsewhere. From the variable velocities obtained in normal and nephrectomized dogs, a considerable variation was to be expected in the fraction of penicillin inactivated extra-renally.

The rate of elimination of penicillin after various surgical procedures

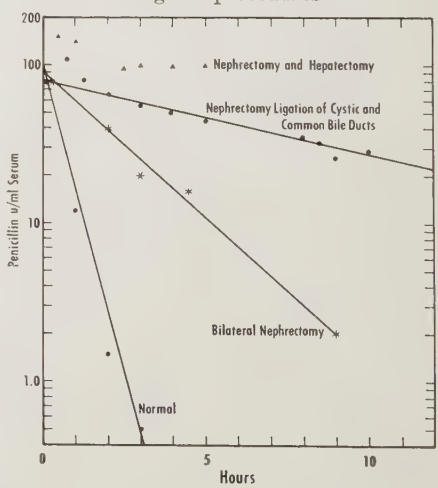

FIG. 2.

All dogs were given 25,000 units of penicillin per kilogram in a single intravenous injection.

Site of the Extra-Renal Elimination. The rate of elimination was not significantly altered by the removal of the intestine from the ligament of Treitz to within an inch of the anus; nor by the removal of the stomach and ligation of the common bile duct. This showed that in these dogs the intestinal tract was not the primary site of the extra-renal elimination, as suggested by the findings of Reid.[3]

Following complete evisceration the serum penicillin concentration remained constant, within the limits of experimental error; thus the site of the elimination was somewhere in the viscera.

A two stage hepatectomy and bilateral nephrectomy were performed in a dog. Here too, no inactivation could be shown after the equilibration of penicillin between the blood stream and the tissues. In another dog, both kidneys and all the viscera except the liver were removed. The hepatic artery was left intact by dissecting away the hepatico-duodenal ligament. The portal vein and the common bile duct were ligated and the gall bladder left in connection with the biliary system. This dog eliminated penicillin at a rate very similar to the dogs in which only bilateral nephrectomy was done. It could be concluded that the liver was responsible for the extra-renal elimination of penicillin by these dogs. The velocity constants indicating the relative decrease in penicillin concentration, or total amount of penicillin per hour, are shown in Table II. The velocity constants were found from the curves by the formula:

$$K = \frac{1n\ (c_{t1}/c_{t2})}{t_1 - t_2}$$

Mechanism of Hepatic Elimination of Penicillin. In 2 dogs the kidneys were removed, the cystic and common bile ducts were ligated twice and cut. Similar curves were found in both dogs; one curve is shown in Fig. 3. It was noted that a certain elimination of penicillin seemed to occur during the first 10 hours; however, the rate of elimination decreased after this to a very low value. Thus, penicillin was still present in appreciable amounts 70 hours after the intravenous injection. The final rate of elimination was only 4% of the normal rate. This finding seemed to indicate that the penicillin was excreted in the bile rather than destroyed by the liver. If so, the initial drop in serum concentration might be due to excretion of bile into the biliary system to the point of distention. However, it still appeared possible that destruction might have taken place in the liver, but that liver function was impaired by the biliary obstruction.

In another nephrectomized dog, the common bile duct was cannulated and the secreted bile collected hourly after an intravenous injection of penicillin. Very high concentrations of penicillin were found in the bile samples. The total amount recovered in the bile was 80% of the injected penicillin. It could be

TABLE II.
The Velocity Constant Indicating the Relative Decrease in Penicillin Concentration in the Serum After Various Surgical Procedures.

Procedure	K 1/hr	Avg
Normal dogs	1.85 1.90 2.40 1.90 1.25 1.15 1.45	1.70
Nephrectomized	0.46 0.38 0.40	0.41
Nephrectomized and the intestine removed from the ligament of Treitz to within one inch of the anus	0.33 0.35	0.34
Nephrectomized, bile duct ligated and complete gastrectomy	0.40	
Eviscerated, except for the liver	0.53	
Complete evisceration	0.0	
Hepatectomy, nephrectomy	0.0	
Cystic and bile ducts ligated; nephrectomized	0.07	
Bile duct cannulated —excreted in bile—	0.31 0.25	
Inactivation in bile at —log cH+ 8.5, 40°C	0.10	

[3] Reid, R. D., Felton, L. C., and Pittroff, M. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 438.

concluded that, at least in this dog, the main part of the extra-renal elimination took place as excretion in the bile.

Inactivation by bile in vitro. A sample of dog's bile (pH 8.29) obtained from a common duct fistula, was mixed with penicillin and incubated at 37°C. A rather slow inactivation was found to take place. Another sample drawn from the gall bladder (pH 6.00) did not inactivate penicillin at a demonstrable rate. This suggested that the inactivation was due to the alkalinity of the fistula bile. The rate of inactivation to be expected in this case was calculated[4] and found to be consistent with the experimental values (Fig. 4). The rate of inactivation in the bile can thus be calculated at known values of temperature and hydrogen ion concentration on the basis of the known destruction rate by alkali.

The slow elimination after bilateral nephrectomy and ligation of cystic and common bile ducts

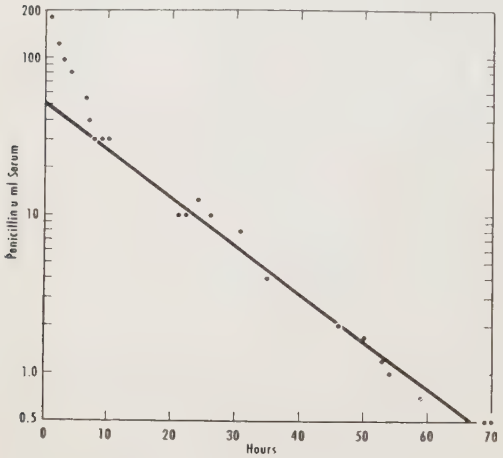

FIG. 3.

The elimination during the first 10 hours may be due to excretion of penicillin-containing bile into the biliary system to the point of distention.

The slower inactivation throughout the 70 hours of the experiment can be explained by the destruction of penicillin due to the alkalinity of the bile.

The inactivation by bile *in vitro*

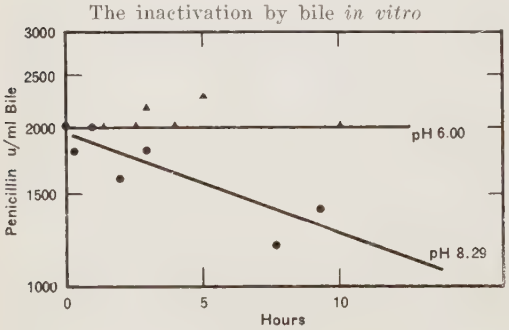

FIG. 4.

The lines show the inactivation, at the given pH values as calculated from the data of Brodersen,[4] in good agreement with the experimental points.

The two samples of bile were obtained from the gall bladder (pH 6.00) and from a common duct fistula (pH 8.29).

The pH values were determined electrometrically by comparison with 0.01 m HCl + 0.5 m NaCl (pH 2.00). The hydrogen ion concentrations were found to be independent of temperature in the range of 20 to 40°C.

Bile and serum penicillin concentration after an intravenous injection of penicillin in a nephrectomized dog

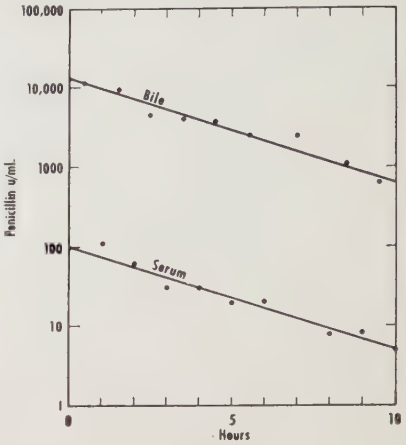

FIG. 5.

The bile is seen to contain about 130 times as much penicillin per milliliter as the serum, independent of the serum concentration.

Relationship of Penicillin Concentrations in Serum and Bile. In Fig. 5 are plotted the concentrations of penicillin in cannulated bile and in blood serum taken simultaneously from a nephrectomized dog. There was a constant ratio between the two concentrations, the bile containing 130 times as much penicillin per ml as the serum at serum concentrations ranging from 100 to 5 units/ml. This ratio was found to vary considerably. In 7 experiments carried out in two dogs, values ranging from 10 to 330 were obtained. From the figures in Table III it is seen that the high ratios generally were found at high pH values in the bile and the low ratios at the low pH values.

[4] Brodersen, R., *Trans. Farad Soc.*, 1947, **43**, 351.

TABLE III.
Correlation Between Bile pH and the Bile Penicillin-serum Penicillin Ratio.

	Penicillin concentration of bile
pH of bile	Penicillin concentration of serum
8.65	330
8.50	130
8.48	270
8.29	10
8.20	16
8.04	11
7.93	11

Storage of Penicillin in the Gall Bladder. A cannula was placed in the gall bladder of a normal dog, and the common bile duct ligated in order to close the outlet from the gall bladder. The bile was drawn from the gall bladder, mixed with 100,000 units of penicillin and re-injected. The volume of bile in the gall bladder was determined every hour and a small sample taken out for penicillin assay. The total amount of penicillin found in the gall bladder is shown in Fig. 6.

Resorption and inactivation of penicillin in the gall bladder

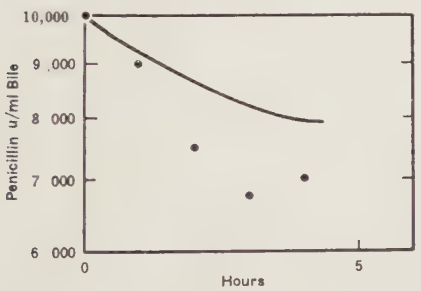

FIG. 6.

The curve indicates the calculated inactivation. The experimental points are seen to deviate relatively little from this curve, indicating that only a slow resorption of penicillin takes place from the gall bladder.

The pH values were determined at the same time and from these the spontaneous inactivation calculated as shown by the curve. The difference between this curve and the points demonstrate the resorption from the gall bladder. Within the 4 hours of the experiment the resorbed amount is seen to be rather small. That a certain resorption does take place was shown by the fact that penicillin was present in the urine of the dog.

Summary. Bilaterally nephrectomized dogs eliminated penicillin G at a considerable rate from their blood stream after an intravenous injection. This elimination took place at essentially the same rate when either the intestines were removed, or when the common bile duct was ligated and the stomach removed. But no inactivation could be demonstrated after complete evisceration or hepatectomy in nephrectomized animals. A major portion of the injected penicillin was excreted in the bile. Appreciable inactivation was found in the bile, especially in the case of a strongly alkaline bile.

The remaining portion of penicillin may have gone either to the gall bladder, where only a slow but significant resorption took place, or to the intestine where it was partially resorbed and partially inactivated.[5]

Caution must be exercised in drawing generalized conclusions from these results. Since the nephrectomized dogs do not eat, it seems possible that normal dogs might inactivate certain amounts of penicillin in their intestine because of a greater secretion of digestive juices.

[5] Seeberg, V. P., Illg, P. I., Brown, D. J., *J. Am. Pharm. Assn.*, 1946, **35**, 280.

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17283. An Immunologic Comparison of Twelve Strains of *Cryptococcus neoformans* (*Torula histolytica*).

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The results of various authors concerning the immunogenic properties of *Cryptococcus neoformans* have not been in agreement.[1-7] Some investigators have been unable to demonstrate antibody formation in animals[4,7] although others have reported agglutinin titers ranging from 1:9[5] to 1:280.[3] Benham[1] obtained serum with an agglutinin titer of 1:160 against pathogenic strains by injecting capsule free cells into rabbits. Working with pathogenic and non-pathogenic strains she has divided the genus Cryptococcus into 4 groups on the basis of serologic and morphologic characteristics. The present investigation is concerned only with strains isolated from human infections (Benham Group III).

Methods. Rabbits were immunized with encapsulated, formalin-killed cells of *C. neoformans*. Intravenous injections were made on three consecutive days of each week. Nine strains of *C. neoformans* were employed for immunization: strains BT, RE, DU, 1523, RO, L2, and LE from the Los Angeles County General Hospital and strains 732 and 2526 from the American Type Culture Collection. Three additional strains were included in the agglutination tests: HA,† BE,‡ and ATCC 4189.

TABLE I.
Agglutination* in Serum Absorbed with Cells of Heterologous Type.

		Serum		
Type	Cells	Type A (RE)†	Type B (1523)‡	Type C (LE)§
A	732	++++	—	—
	2526	++	—	—
	4189	++	—	—
	BT	++++	—	—
	RE	++++	—	—
	DU	++++	—	—
	BE	++++	—	—
	HA	++++	—	—
B	1523	—	+++	—
	L2	—	+++	—
	RO	—	++	—
C	LE	—	—	++++

* Microscopic slide technic; serum dilution 1:20.

† Absorbed with strain L2. Similar results were obtained when other Type B strains were used for absorption. (See Table II).

‡ Absorbed with strain RE. Similar results were obtained when other Type A strains were used for absorption.

§ Absorbed with Type B cells.

1 Benham, R. W., *J. Inf. Dis.*, 1935, **57**, 255.

2 Drake, C. H., *Proc. Soc. Am. Bact.*, 1948, **1**, 57.

3 Hoff, C. L., *J. Lab. and Clin. Med.*, 1942, **27**, 751.

4 Kligman, A. M., *J. Immunol.*, 1947, **57**, 395.

5 Neil, J. M., Castillo, C. G., Smith, R. H., and Kapros, C. E., *J. Exp. Med.*, 1949, **89**, 93.

6 Rappaport, B. Z., and Kaplan, B., *Arch. Path. and Lab. Med.*, 1926, **1**, 720.

7 Sheppe, W. M., *Am. J. Med. Sci.*, 1924, **167**, 91.

† Obtained from Dr. M. Marples, Otago University Medical School, New Zealand.

‡ From the Los Angeles County General Hospital.

TABLE II.

Agglutination* in Type A (RE) Serum after Absorption with Homologous and Heterologous Cells.

Cells	Unabsorbed serum	Serum absorbed with: 732	2526	4189	BT	RE	DU	BE	HA	1523	L2	RO	LE
732	+									+	+	+	+
2526	++									++	++	++	++++
4189	++									++	++	++	++++
BT	++									+	+	+	+
RE	++++									+++	+++++	+++	+++
DU	++++									+++	+++++	+++	+++
BE	++++									++	++++	++	++
HA	++++									++	++++	++	++
1523	++												++
L2	++												++
RO	±												±
LE													

* Microscopic slide technic; serum dilution 1:20.

Agglutinations were performed by the serial dilution tube test and by a microscopic slide technic using serum dilutions of 1:20.

After twelve weekly series of injections agglutinating titers of 1:320 were obtained against strains DU, RE, and 1523. Strain LE produced a titer of 1:40.* The other strains with the exception of RO and ATCC 732 had developed low titers (1:10 to 1:40) at the end of 12 weeks.

Agglutinin absorptions were conducted by the method of Krumwiede, Cooper and Provost.[8]

Results. From the data presented in Table I, it is evident that serologic differences exist among the strains employed. For convenience, the 3 varieties have been designated as Types A, B, and C. With the exception of anti-DU serum, it was necessary to absorb all serum with heterologous cells to prevent cross-reactions. The anti-DU serum agglutinated all Type A strains to a titer of 1:320, but failed to agglutinate Types B and C. Table II presents the results of slide agglutinations in anti-RE (Type A) serum following absorption with homologous and heterologous cells. These data were confirmed by similar absorptions of Type B (1523) and Type C (LE) sera.

Discussion. There appears to be a difference in the immunogenic properties of different strains of *C. neoformans*. Although the rabbits used in the present investigation received equal numbers of killed cells, high titers were produced against only 3 strains. This variation may account for the failure of some authors to elicit antibody formation.

Although it is obvious that there are differences in the antigenic structure of the strains used here, no conclusions can be drawn regarding the location of the antigens responsible. It seems possible that the capsular carbohydrate may be important since Neil and his associates[5] have reported a polysaccharide

* Although this serum is of considerably lower titer than the others, it was included in the reciprocal agglutination tests because strain LE appeared to be heterologous to all other strains used.

[8] Krumwiede, C., Cooper, G., and Provost, C. J., *J. Immunol.*, 1925, **10**, 55.

from Cryptococcus which was serologically active up to a dilution of 1:2,000,000.

Summary. 1. Rabbits were immunized with 9 strains of *Cryptococcus neoformans*. After 12 weekly series of injections, 3 strains produced agglutinin titers of 1:320, 4 strains gave titers ranging from 1:10 to 1:40 and 2 strains failed to produce antibodies.

2. On the basis of reciprocal agglutinin absorptions, 3 serologic types of the organism are described. These have been designated as Types A, B, and C.

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17284. Electron Microscopy Study of Chick Embryo Erythrocytes.

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A few electron micrographs of erythrocytes are found in the literature.[1-7] Erythrocytes with cytoplasm made transparent to electrons apparently by the hypotonic solutions which were used in specimen preparation were discovered accidentally in preparations of isolated liver cell nuclei which were being examined in an electron microscope.

Materials and methods. Nuclei of liver cells from healthy, 16-day-old chick embryos were isolated by the differential centrifugation technic of Dounce[8] as modified by Hoerr,[9] and a portion of the final sediment was resuspended in distilled water for immediate deposition upon electron microscope specimen screens. Three other portions were resuspended for ten minutes in 10% formalin, in 2% osmic acid and in 1% silver nitrate, respectively, and specimens of each were prepared as before. The proportion of reagent volume to specimen volume was about 10:1 and seemed excessive, but smaller proportions of reagent and less time for reagent action gave bad results also. Some of the prepared screens which were formalin-fixed only were stained with Harris hematoxylin for 5 minutes; others with 1% safranin for one minute and still others with 1% methyl green for one minute and were washed with distilled water to remove the excess stain. Wet preparations were examined with the ordinary light microscope for control purposes.

Micrographs were taken employing a biased electron gun and an objective aperture with relatively low plate magnifications between 3,000 and 4,000 times. Crystalline residues, which are recognizable in electron micrographs were often observed, and examples of some are indicated at the arrows in Fig. 4, 5 and 12. No effects of electron bombardment were noted upon the morphology of the cells, except that they shrunk when focussed beams were directed upon them, but none of the fields reproduced here have been subject to focussed beams.

Observations and discussion. Unfixed, unstained erythrocytes are identified in this work by their large size, almost filling the whole 2 inches square field at 3,600 times; by their characteristic oval shape; by the centric position and the diffuse, elliptical outline of the nucleus within them; by the proportion of their cytoplasmic to nuclear area; by the homogeneity of their cytoplasm and

1 Wolpers, C., *Naturwiss.*, 1941, **29**, 416.

2 Jung, F., *Klin. Wochsch.*, 1942, **21**, 917.

3 Rebuck, J. W., and Woods, H. L., *Blood*, 1948, **3**, 175.

4 Rebuck, J. W., Woods, H. L., and Monaghan, E. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 220.

5 Barnes, R. B., Burton, C. J., and Scott, R. C., *J. Appl. Phys.*, 1945, **16**, 730.

6 Jones, W. M., *J. Sci. Instr.*, 1947, **24**, 113.

7 Heinmetz, F., *J. Bact.*, 1948, **55**, 823.

8 Dounce, A. L., *J. Biol. Chem.*, 1943, **147**, 685.

9 Hoerr, N. L., *Biol. Symposia*, 1943, **10**, 185.

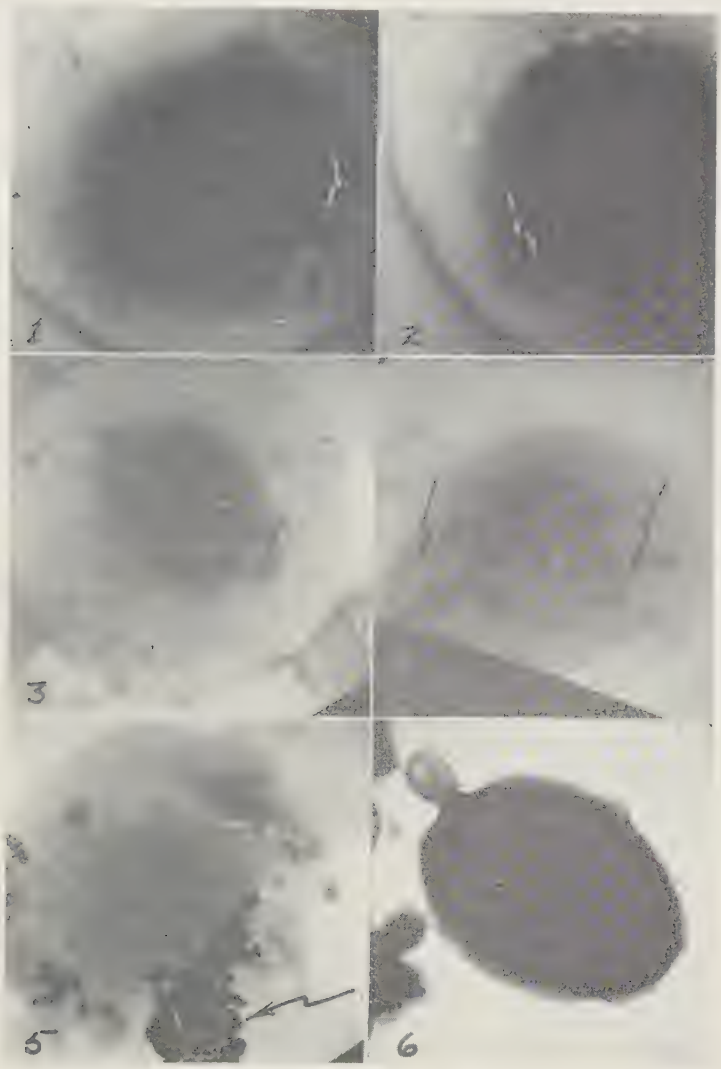

PLATE I.
Electron micrographs of osmosis-hemolyzed chick embryo erythrocytes. × 4000.
Fig. 1, 2, 3, 4 and 5: Unfixed and unstained. Salt residues indicated at arrows.
Fig. 6: Osmic acid fixed where fixation seems to be excessive.

lack of nucleoli in their nuclei; and by the high degree of permeability to electrons of the cytoplasm over the nucleus. They are easily differentiated further from isolated nuclei by their outlines, for where those of the erythrocytes are sharp and well-distinguished, those of the nuclei are diffuse and indistinct.

The nucleus appears clearly in these erythrocytes, except where they have been osmic acid or silver nitrate fixed when detail over almost the whole body of the cell is lost. The nucleus has normal oval appearance in the unfixed, unstained cells and is centric, diffuse-edged and reticular in structure. It shrinks and deforms when it has been formalin-fixed and no structure is seen in it.

Several degrees of hemolysis were apparently provoked by the hypotonic solutions employed in the preparation of the material. Thus, there are different degrees of opacity of the cytoplasm to electrons in each of Fig. 1, 4

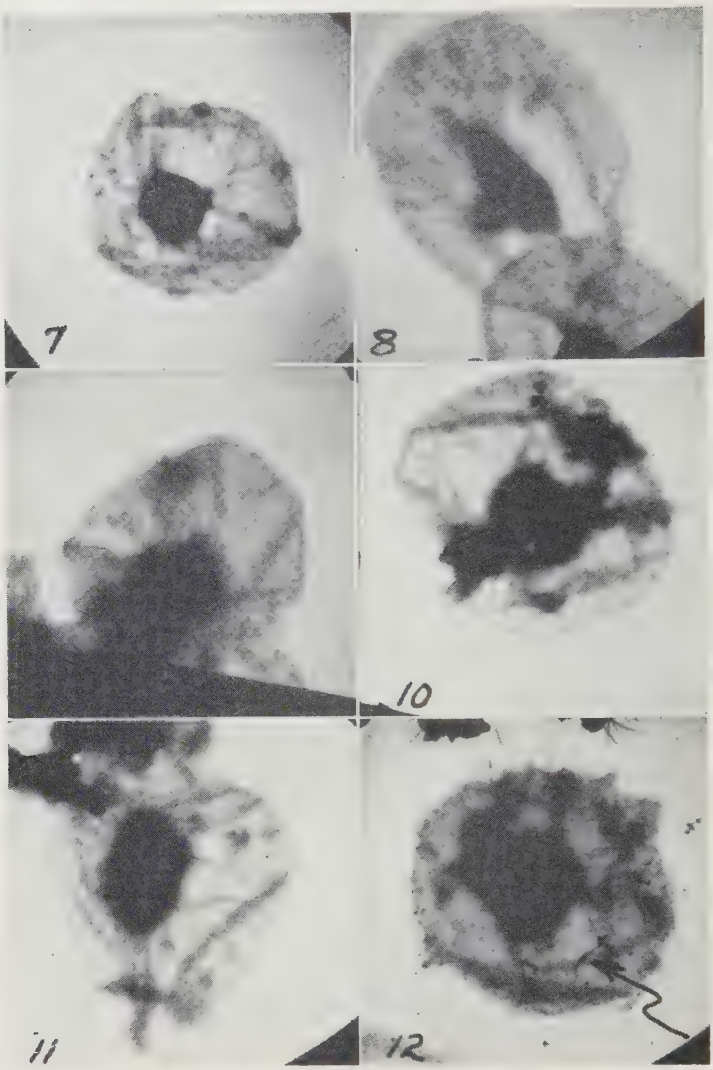

PLATE II.
Electron micrographs of osmosis-hemolyzed, chick embryo erythrocytes, formalin fixed. × 3630.
Fig. 7, 8 and 9: Unstained
Fig. 10: Hematoxylin stained.
Fig. 11: Methyl green stained.
Fig. 12: Safranin stained.

and 5, but the cytoplasm of each of the hemolyzed erythrocytes in Fig. 1 to 5, inclusive, is somewhat transparent to electrons as compared with the known homogeneous opacity of unhemolyzed erythrocytes.[3,4] The sponge-like appearance of the erythrocytes in Fig. 4 and 5 may be the result of an incomplete hemolysis, but it could also support the theory that erythrocytes have a spongy structure. On the other hand, appearance of the images in Fig. 2, 3 and 7 to 12 would support the theory of a balloon structure for erythrocytes.

On several occasions it was possible to rupture the films which supported the erythrocytes and to observe their cross-sections as they were tilted upon them. Under these circumstances the well-hemolyzed erythrocytes

(Fig. 1, 2 and 3) were seen to be extremely flat with outline reminiscent of that of a fried egg. From considerations of electron scattering from such shapes it is very unlikely that the dark outlines in Fig. 1, 2 and 3 can be interpreted as arising from a cell wall or a cell membrane. On the contrary, the most probable explanation for the dark outline and for its non-uniform thickness is that they arise from adsorbed or other material caught about the edge of the cell area as it dries. However, the fact that there is a discrete cell area at all in the micrographs indicates that a cell membrane may exist, although it is invisible in the micrographs. The dark outlines are not visible in the partially hemolyzed or the fixed erythrocytes although the cell areas are again quite discrete. This is evidence, coupled with the previous observations that neither the partially hemolyzed nor the fixed cells flatten out on the films to the same extent that the well-hemolyzed erythrocytes do, and it also suggests again that a cell membrane exists for an erythrocyte, but that it is thin enough physically and atomically to produce negligible electron scattering. Further evidence for the existence of a membrane is given later in the discussion on fixed erythrocytes.

The observations cited so far concerning hemolyzed and partially hemolyzed, unfixed erythrocytes tend to support the important concept of erythrocyte structure, that there is a cell membrane. They also support the following hypotheses concerning the mechanism of erythrocyte hemolysis: (1) that at partial hemolysis an amount of hemoglobin leaves the cell sufficient to allow some electron transmission, so that a spongy appearance becomes manifest over the cell area; at this time (Fig. 4 and 5), no cell membrane is seen, the cell is still distended and a nucleus is dimly visible, and (2) that at the more advanced stages of hemolysis the spongy content contracts upon the nucleus increasing its opacity but leaving a clear area around it, the cell flattens due to lack of cell content and adsorbed material collects about the edge of the cell area as it dries.

In spite of the obvious distortions which are introduced by the fixation (see below) erythrocytes in fixed preparations are recognized and distinguished from the nucleus by use of the same criteria as they are in the unfixed samples. No significant, morphologic differences are detected between erythrocytes fixed with formalin but not stained and those fixed with formalin and stained with hematoxylin, methyl green or safranin. Fig. 10, 11 and 12 show erythrocytes which were fixed and stained with a variety of stains. Fig. 7, 8 and 9 are of a formalin-fixed, unstained sample. Erythrocytes in fixed specimens, both stained and unstained, are obviously distorted due chiefly to the fixation, since unfixed cells are not observed to shrink in the electron microscope at the low intensities at which these were examined. Marked shrinkage of the erythrocytes was also noted in the fixed specimens examined with the light microscope where there was no drying.

Except for the existence of a nucleus, fixed erythrocytes resemble empty bladders in electron micrographs (Fig. 7 to 12), and are similar in this respect to the non-nucleated erythrocytes studied by Wolpers[1] and Jung.[2] The fixed erythrocytes show foldings which are frequently radiated and which indicate that the erythrocytes possess an envelope or membrane. In mammalian erythrocytes at least, this envelope apparently corresponds to the plasma membrane with very little cytoplasm.[10] Such foldings would not be easily seen and indeed are not observed in wet preparations examined with the optical microscope, although they might exist in the wet. It seems logical to explain the occurrence of folds in electron micrographs by assuming that the fixed cell is flabby and its envelope loose. In the act of drying, the envelope falls together on the surface of the specimen film and the folds become visible. In the recently reported work of Chu, Dawson and Elford[11] electron micrographs of fixed chicken red cells are shown which exhibit morphology similar to that reported here.

The reticular structure of the stretched erythrocyte envelope depicted by Wolpers[1]

[10] de Robertis, E. D. P., Nowinski, W. W., and Saez, F. A., General Cytology, Philadelphia, W. B. Saunders Co., 1948, 122.

[11] Chu, C. M., Dawson, I. M., and Elford, W. J., *Lancet*, 1949, **CCLVI**, 602.

offers a striking similarity to the cytoplasmic structure of some of the hystiocytes described by Rebuck and Woods.[3] Wolpers and Ruska[12] have reported a similar structure for the cytoplasm of the blood platelet hyalomere. One wonders about the relation of fixation and other technical processes to these appearances because in an electron microscope study of tissue culture cells, Porter, Claude and Fullam[13] observed a remarkable variation in the appearance of cytoplasm structure according to the fixation technic employed. Frey-Wyssling[14] points to the possibility that Wolpers' images show artifacts since a similar structure has been observed in the erythrocyte envelope denatured by heat hemolysis.[2]

The outlines of fixed erythrocytes are often observed to be angular. In Fig. 9 the outline is almost hexagonal and approximations to this particular shape were often encountered. The nuclei of fixed erythrocytes are also shrunken and distorted markedly, and are somewhat angular in outline. Nucleus distortions similar to those in the figures of Pl. II were observed in the control wet preparations. This suggests again that the causes of such distortion are to be found not as much in the act of drying as in the action of the fixative on cells which have previously suffered osmotic changes, since the nuclear distortions like the whole-cell distortions are not seen in fresh preparations when the sample is unfixed and osmotic changes have not occurred. The opacity of the nucleus to electrons is increased by the fixation, the contrast vis-a-vis nucleus and cytoplasm being improved thereby. However, this improvement in contrast offers no additional morphological information over unstained specimens; rather, detail is lost due to the increase in opacity of the nucleus. In most of the unfixed as well as the fixed erythrocytes, the nucleus is too opaque to electrons to evidence its structural details, but a coarse reticulum can be observed in some of them in the unfixed samples, Fig. 3 and 5.

A reliable identification of erythrocytes could not be made in specimens which had been treated with either osmic acid or silver nitrate. Both fixatives produced almost homogeneously opaque, oval images in which a reticular structure was evidenced but only at the borders. These erythrocytes had also shrunk and the most marked shrinkage was observed in those treated with osmic acid.

Besides the observations of morphology which have been made in this work and reported in the foregoing discussion, there are a number of points concerning the electron microscopy of biological material[15] which have been illustrated: (a) a biased gun, if used properly and at less than maximum intensities does not affect the subject more than an unbiased one; (b) the primary heating effect of the electron beam is to shrink the cells, but this shrinkage from actual observation is not as drastic, nor does it introduce so many artifacts as does the process of fixation under the experimental conditions; (c) the processes of fixing and staining here tended to complicate the interpretation of electron microscope images rather than to simplify or clarify it; and (d) in general, electron images were likely freer of artifact than light microscopic images when unfixed, unstained samples were used in the former and fixed, stained samples in the latter.

Summary. Electron micrographs of osmosis-hemolyzed erythrocytes from healthy chick embryos are presented. The cells are examined unfixed, unstained; formalin-fixed, unstained; formalin-fixed, hematoxylin stained; formalin-fixed, safranin stained; formalin-fixed, methyl green stained; osmic acid fixed, unstained; and silver nitrate-fixed, unstained. Unfixed, unstained erythrocytes prepared by the technic employed showed a transparent cytoplasm so that the direct visualization of intracytoplasmic parasites might be possible by this procedure. Some nuclear structure was observed, and there is evidence for the existence of a cell membrane. Fixed erythro-

[12] Wolpers, C., and Ruska, H., *Klin. Wochsch.*, 1939, **18**, 1077, 1111.

[13] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

[14] Frey-Wyssling, A., Submicroscopic Morphology of the Protoplasm and its Derivatives, New York, Elsevier Publishing Co., Inc., 1948, 173.

[15] Watson, J. H. L., *J. Appl. Phys.*, 1948, **19**, 713.

cytes clearly showed an envelope. Fixation and drying produced specimen changes, and in the specimens examined the processes of electron microscopy provoked less radical changes than did the fixation.

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17285. Effect of Thymine Desoxyriboside (Thymidine) on Human Pernicious Anemia.

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Shive *et al.*[1] isolated from liver a crystalline substance that inhibited the antagonistic action of methyl-folic acid on the growth of *Leuconostoc mesenteroides* 8293. This substance was identified as thymidine, the desoxyriboside of thymine. Wright *et al.*[2] reported that thymidine could replace vitamin B-12 as a growth factor for certain lactic acid bacteria. Hypoxanthine, adenine or cytosine desoxyribosides[3] and guanine desoxyriboside[4] have also been shown to be able to replace vitamin B-12 as a growth factor for various bacteria.

From this evidence it seemed possible that vitamin B-12 may participate in the synthesis of desoxyribosides, essential to the formation of desoxyribose nucleic acids. Since thymine is effective in combating the hematologic lesions of pernicious anemia, nutritional macrocytic anemia and sprue,[5] the effect of thymidine on patients with pernicious anemia was investigated.

Methods. Three patients with Addisonian pernicious anemia in relapse were treated. All of them had a macrocytic, high color index anemia, with gastric achlorhydria after histamine and megaloblastic hyperplasia of the bone marrow. Reticulocyte counts were done daily, and erythrocyte counts twice a week during the period of observation.

Results. In the first patient, a single injection of 5.3 mg of thymidine was followed by an increase of reticulocytes from 2.0 to 5.0% 4 days later, but by no significant rise in erythrocytes. One week after administration of the thymidine, daily intramuscular injections of 0.001 mg of vitamin B-12 were begun. There was a reticulocyte rise to 10.3% and the red blood cells rose from 2,430,000 to 4,250,000 in the next 13 days.

In the second patient a single injection of 150 mg of thymidine was followed by a rise in reticulocytes from 0.4% to 5.3% on the 4th day, but the red count failed to rise during the week following the injection. Daily intramuscular injections of 0.001 mg of vitamin B-12 were then started. Reticulocytes were 37% on the 7th day and the blood count had risen from 1,600,000 to 4,200,000 on the 21st day of this therapy.

The third patient received a sub-optimal intramuscular dose of 0.00025 mg of vitamin B-12 daily. Reticulocytes increased to 15.5% on the 8th day, and were 1.8% on the 17th day of therapy. At this time, in addition to the vitamin B-12, 5 mg of thymidine was given intramuscularly daily for 9 days. There was a secondary reticulocyte rise from 0.8% on the 18th day to 2.8% on the 21st, 22nd,

* Died May 20, 1949.

1 Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, 1948, **70**, 2299.

2 Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

3 Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, 1949, **177**, 993.

4 Hoff-Jorgensen, E., *J. Biol. Chem.*, 1949, **178**, 525.

5 Spies, T. D., and Stone, R. E., *Lancet*, 1947, **1**, 174.

and 24th days. Injections of 0.00025 mg of vitamin B-12 were continued from the 27th to the 40th day. During the first period of vitamin B-12 alone the red blood cell count rose from 1,850,000 to 2,220,000. During the second period with thymidine added the count increased further to 2,800,000. At the end of the third period of sub-optimal vitamin B-12 alone it had decreased to 2,600,000.

Discussion. The replacement ratio of thymidine for vitamin B-12 is about 300:1 for the growth of *L. lactis*.[6] We have seen a maximal reticulocyte response in pernicious anemia to as little as 4 μg of B-12.[7] A dose of 5 mg of thymidine should therefore be more than adequate if the ratios observed in the bacterial growth system obtain for man. (That this is so for thymine with reference to folic acid was shown by Spies and his associates.[5]) From the results we obtained, it appears that thymidine alone is incapable of sustaining blood regeneration, although it does appear to cause a slight increase in reticulocytes. Similar results were reported by Geerts and Lens using doses of 10 mg of thymidine a day for 3 days.[8] From our observations it is apparent that the response to 150 mg was no greater.

It has been suggested that crude liver extracts may contain substances that give an enhanced hematopoietic response compared to B-12, and thymidine may be one of these accessory substances.[9] From our study it appears that thymidine is not completely inert hematopoietically, but if it has an enhancing effect on B-12 activity, this effect is very slight in the dosage used in this study. In view of the ability of other desoxyribosides to substitute for B-12 in bacterial growth, the effect of mixtures of desoxyribosides on pernicious anemia should be investigated.

Summary. 1. In 3 patients with pernicious anemia, thymidine in doses of 5 to 150 mg was found to cause slight reticulocytosis, but no effect on the red blood count.

We are deeply indebted to Dr. Esmond E. Snell, who generously supplied us with the thymidine used in these studies, and Mr. W. S. McNutt, who prepared it.

6 Shive, W., Ravel, J. M., Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

7 West, R., and Reisner, E. H., Jr., *Am. J. Med.*, 1949, **6**, 643.

8 Geerts, S. J., and Lens, J., *Nature*, to be published.

9 Jacobson, M., and Bishop, R. C., *J. Clin. Invest.*, 1949, **28**, 791.

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17286. Effect of Adrenalectomy on Eosinophil Response of Rats Infected with *Trichinella spiralis*.

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Recently there have appeared reports[1-4] that adrenal hormones depress the number of circulating eosinophils. Injection of adrenal cortical hormone will cause a lowering of the number of circulating eosinophils, or injection of adrenotrophic hormone from the pituitary will accomplish the same result if the adrenal gland is intact. In fact, this lowering of the eosinophils taken together with other changes in the body chemistry is proposed as a test for adrenal cortical insufficiency. Presumably the presence of the adrenal cortex has an inhibitory influence on the eosinophil producing bone marrow.

The eosinophil response to infection with *Trichinella spiralis* usually is striking in

1 Thorn, G. W., Forsham, P. H., Prunty, F. T., and Hills, A. G., *J. Am. Med. Assn.*, 1948, **137**, 1005.

2 Forsham, P. H., Thorn, G. W., Prunty, G., and Hills, A. G., *J. Clin. Endocrinol.*, 1948, **8**, 15.

3 Hills, A. G., Forsham, P. H., and Finch, C. A., *Blood*, 1948, **3**, 755.

4 Hellman, L., *Science*, 1949, **109**, 280.

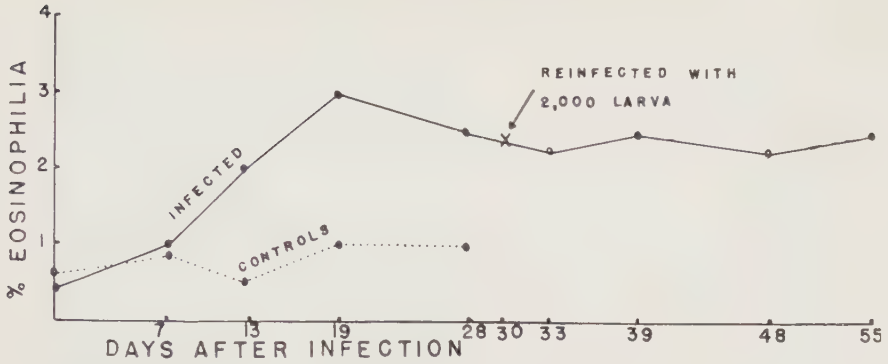

FIG. 1.

Eosinophil response of intact rats infected with *Trichinella spiralis* larvae, as compared with intact uninfected controls.

humans (Gould[5]) and has been reported rather high for mice (Hunter and Groupé,[6] Stein[7]) and rats (Someren[8]). This suggested that the eosinophil response in intact and adrenalectomized animals infected with this parasite would yield interesting results. Thus the following study was undertaken.

Experimental. Preliminary experiments were performed to check the eosinophil response of our rats to *T. spiralis* infection. These animals (Wistar strain) were raised and maintained on our stock diet in an air-conditioned room (76°F). The experimental procedures for infecting the animals with standard doses of larvae have been described elsewhere (Larsh and Kent[9]). It is important to point out that the viability of these larvae in all experiments was checked by determining in a few control animals the percentage development of adult worms 5 days post-infection. The blood for eosinophil counts was obtained from the tail, stained with Giemsa stain in the usual manner, and examined under oil immersion. These counts were done at approximately weekly intervals.

Sixteen rats (2.5 months old) were used to determine the eosinophil response. Three received 500 larvae; three, 1000 larvae; three, 1500 larvae; three, 2500 larvae; and 4 were kept as uninfected controls. The infected animals were reinfected with 2000 larvae 30 days after initial infection. Since the eosinophil response was similar for all of the infected animals, the results were plotted as the same (Fig. 1). The graph shows very little eosinophilia produced in these animals, as contrasted to the 20% (in 16 days) mentioned above (Someren[8]). Our results, however, compare favorably with those of Beahm and Downs.[10] It is unlikely that our results were due to a strain factor in the rats in that random counts of another rat strain infected as above revealed about the same findings. It is possible that our strain of *T. spiralis* is a poor stimulator of eosinophilia. There is some support for this in that our white mice (2.5 months old), likewise, showed a poor eosinophil response. These were infected with various doses of *T. spiralis* larvae; 3 with 50 larvae; 3, 100 larvae; 3, 300 larvae; and 3, 400 larvae. All of these showed about the same level of eosinophilia, which reached a maximum of 10% after one month. The 4 non-infected controls averaged about 3%. The response of the infected mice was considerably lower than that (25-33% within 2 weeks) reported by the above mentioned workers.[6,7]

Despite the poor eosinophil response described above, it was decided to determine

5 Gould, S. E., Trichinosis, 1945, C. C. Thomas Co., Springfield, Ill.

6 Hunter, G. W., III, and Groupé, V., *J. Parasitology, (supp.)*, 1939, **25**, 33.

7 Stein, K. F., *Anat. Rec.* 1949, **103**, 508; PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 225.

8 Someren, V. D., *J. Helminth.*, 1938, **16**, 83.

9 Larsh, J. E., Jr., and Kent, D. E., *J. Parasitology*, 1949, **35**, 45.

10 Beahm, E. H., and Downs, C. M., *J. Parasitology*, 1939, **25**, 405.

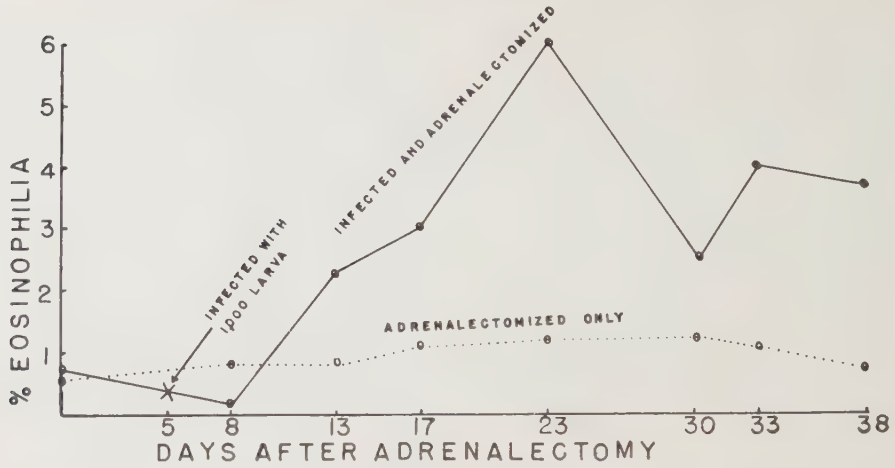

FIG. 2.

Eosinophil response of rats infected with *Trichinella spiralis* larvae 5 days after adrenalectomy, as compared with uninfected adrenalectomized controls.

the effect on this response of adrenalectomy. This work was limited to rats. Nineteen (2.5 months old) were adrenalectomized in a one stage operation, and kept on a potassium-low diet (Nichols[11]). They were given drinking water with 0.8% NaCl and 0.1% $NaHCO_3$, and remained in apparent good condition throughout the experiment. The completeness of adrenalectomy was verified at autopsy. Eleven of these rats were infected with 1000 *T. spiralis* larvae 5 days after adrenalectomy, the remaining 8 were kept as uninfected controls. The eosinophil counts of all of the animals are shown in Fig. 2. While the counts are somewhat higher than those shown in Fig. 1 for intact rats, the difference is too slight to draw definite conclusions. Perhaps it would be worthwhile to repeat this experiment with an animal which has a significantly higher granulocyte count than the rat.

Summary. (1) White mice and rats failed to show a high eosinophilia following initial infection with various doses of *T. spiralis*. (2) Rats also failed to elicit a striking response following reinfection. (3) Adrenalectomy influenced only slightly the number of circulating eosinophils in infected rats.

[11] Nichols, J., *Arch. Path.*, 1948, **45**, 717.

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17287. Local Sweating in Man Induced by Intradermal Epinephrine.

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The possibility of an adrenergic innervation of the sweat glands was suggested recently by Haimovici,[1] who reported that intravenous injection of neosynephrine was followed by an increase in palmar sweating; this response was inhibited by dibenamine (N,N-dibenzyl-β-chloroethylamine hydrochloride). In preliminary experiments[2] in our laboratory, intradermal injection of neosynephrine into the palm failed to alter the pattern of spontaneous sweating. Patton[3] measured electrical potentials of the cat's paw, as an index of sweat

[1] Haimovici, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 40.

[2] Janowitz, H., Sonnenschein, R. R., and Grossman, M. I., unpublished observations.

FIG. 1a.
The photograph demonstrates the vasoconstriction on the forearm of a subject 7 minutes after intradermal injection of 0.1 cc epinephrine 1:100,000.
FIG. 1b.
A sweat print of the same area as that in 1a 5 minutes after the injection. Note the lymphatic spread downward.

gland activity, and observed no blocking effect of dibenamine. Unfortunately, no tests were made with sympathomimetic drugs; furthermore, the results are not necessarily applicable to man. The present study followed the accidental observation of a local sweat response on the forearm of two subjects after the intradermal injection of epinephrine. After this study was underway the report of Kisin[4] appeared, stating that the subcutaneous injection of adrenaline, in concentrations as low as 10^{-8}, produced local sweating.

Methods. In all experiments, Randall's iodine-starch paper method[5] has been used. Injections of approximately 0.1 cc were made with a 27 gauge needle into the skin of the volar surface of the forearm, except as noted otherwise. Observations were continued for 5 to 20 minutes after injection.

Results. Thirty subjects have been tested of whom nine showed no significant response to commercial, synthetic epinephrine hydrochloride (Winthrop Chemical Company), 1:10,000 or 1:100,000, in physiological saline; 5 of these were tested with acetylcholine or nicotine and gave a marked sweat response. On one occasion epinephrine 1:100,000 caused an apparent inhibition of sweating in an individual who had rather profuse spontaneous sweating. The remaining 21 subjects manifested definite sweating after epinephrine 1:10,000 to 1:1,000,000. The response varied widely in intensity among subjects, and in individual subjects on different days. In several experiments the solutions used were made up with crystalline epinephrine (Adrenalin, Parke, Davis & Co.) rather than from the stock solution, and results were identical. Members of both sexes, and of white, Negro and mongoloid races have been tested; no differences in responses of these groups were apparent.

The response, when present, commenced within a minute of the injection. The pattern of sweating closely approximated the area of vasoconstriction, and followed its local and lymphatic spread (Fig. 1). Secretion was observed up to 20 minutes, at which time the rate seemed to have diminished. Neosynephrine hydrochloride (Winthrop-Stearns) 1:10,000 or 1:100,000 produced sweating in

[3] Patton, H. D., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 412.

[4] Kisin, E. E., *Vestnik Venerol. i. Dermatol.*, 1948, No. **5**, 27. (Abstracted in *Chem. Abstr.*, 1949, **48**, 2323).

[5] Randall, W. C., *J. Clin. Invest.*, 1946, **25**, 761.

4 individuals who responded to epinephrine, but the response was less intense. Three subjects who did not respond to epinephrine also failed to respond to neosynephrine.

Pretreatment of the skin by intradermal injection of 0.4 cc atropine sulfate 1:100,000 (4 subjects) or tetraethylammonium chloride 1:100 (2 subjects) had no significant effect on the response. At these concentrations, atropine has been shown to block the local action of acetylcholine, and TEA its axon reflex effects.[6] Procaine hydrochloride 1:100 (4 subjects), however, caused a slight to marked inhibition. Dibenamine was introduced by ion transfer* into the skin of 2 subjects who had shown marked response to epinephrine. Twenty-four hours later, the treated areas of both subjects showed no response to epinephrine 1:1,000,000, while a definite effect was seen on the control arm. Epinephrine at 1:100,000 was only partially inhibited. The dibenamine had no significant effect on the response to acetylcholine chloride 1:1,000,000.

Comment. The epinephrine effect differs from that of acetylcholine in that the latter is characterized by (1) an associated axon reflex, (2) inhibition by atropine, (3) absence of inhibition by dibenamine.

These results indicate that at least some sweat glands of certain individuals are sensitive to the direct action of epinephrine but the physiological significance of this phenomenon is unknown. Experiments are in progress to elucidate the possible role of adrenergic fibers in regulation of sweat responses. Questions under consideration include the distribution of sweat glands which are sensitive to epinephrine, and their activity during thermoregulatory and emotional responses.

Summary. Intradermal injection of epinephrine caused local sweating in 21 of 30 subjects. The response was not altered by atropine or TEA but was diminished by procaine; dibenamine inhibited it. The physiological significance of this phenomenon remains to be elucidated.

Received May 24, 1949. P.S.E.B.M., 1949, **71**.

[6] Janowitz, H., and Grossman, M. I., *Science*, 1949, **109**, 16.

* 0.25 cc of a 5% solution of dibenamine was applied to an asbestos electrode of 7.5 sq cm. A current, whose density was 0.3 milliamp. per sq cm, was passed for 25 minutes. Performed through courtesy of Dr. Arthur A. Rodriquez, Department of Physical Medicine.

17288. Effect of Feeding Dried Egg Plant (Solanum Melonga L.) on Plasma Cholesterol.*

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In a recent paper Roffo[1] stated that egg plant (Solanum Melonga L.) has a decholesterolizing effect in rabbits as well as in man. He also stated that it causes a diuresis. Hainline[2] has recently presented data which he interprets as showing a like effect in rats.

Roffo has published graphs showing a decrease in serum cholesterol in rabbits. We were unable to find any data regarding his experiments with humans other than the statement that a like effect was noted. We have concluded that Hainline's interpretations are open to question, since a number of factors including trauma and infection were not controlled.

Because of our interest in finding some substance that would decrease the plasma cholesterol it was decided to feed dried egg plant to

* The authors are grateful to the California Vegetable Concentrates, Inc., for furnishing as well as processing the egg plant.

[1] Roffo, A. H., *Yale Jr. Biol. and Med.*, Oct., 1945, **18**, 25.

[2] Hainline, A., Jr., Thesis presented to the faculty of the Graduate College, University of Denver, March 8, 1948.

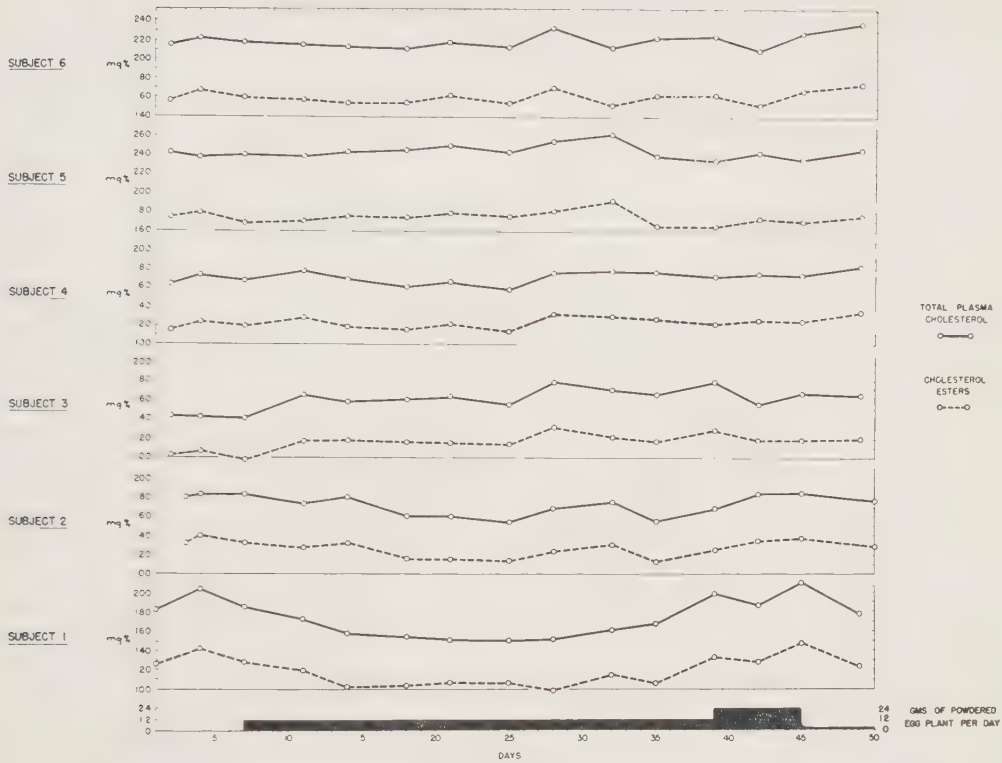

FIG. 1.

The values for both total plasma cholesterol in mg % and esterified plasma cholesterol in mg % are plotted individually for each of the six subjects against a common ordinate which shows the dosage of egg plant administered.

healthy males to determine its effect. One hundred fifty pounds of fresh egg plant was dried in a regular tunnel type drier; *i.e.*, the vegetable material was placed on trays and passed through the tunnel against a stream of air. Two stages of drying were used; *i.e.*, in the first stage a temperature of 145°F was maintained and the moisture content of the vegetable was reduced to approximately 12%. The vegetables were then removed from the trays in the tunnel and put into drying bins. Here the moisture was reduced from approximately 12% to approximately 4% and heat, not in excess of 130°F, was applied. This resulted in 9.5 pounds of slices which were powdered.

This powder was fed in doses of 12 g, and later 24 g a day, to six healthy males. Complete lipid fractionations on the blood plasma were done every third day for a control period of one week and throughout the experiment. The values for free and esterified cholesterol are shown in Fig. 1. The remainder of the blood lipids likewise showed no change.

There were some unavoidable differences in the conditions under which our experiments were conducted and those of Roffo and Hainline. Roffo does not state how his material was dried nor does he give weights before and after drying. Hainline states that his was air dried but does not give weights before and after drying. Due to the pressure of other experiments upon our laboratory we were unable to use the egg plant for several months after it was delivered to us. It was however, hermetically sealed in light and water tight containers and kept in a cool place.

As can be seen, we observed no decholesterolizing effect upon any of the subjects. Subjects 1 through 4 had normal blood lipid values while subjects 5 and 6 are assumed to

have Essential Familial Hypercholesterolemia[3] and to represent the heterozygous abnormal of this condition.

[3] Wilkinson, C. F., Hand, E. A., and Fliegelman, M. T., *Ann. Int. Med.*, Oct., 1948, **29**, 4.

Received June 21, 1949. P.S.E.B.M., 1949, **71**.

17289. Synthesis of Nucleic Acid and Phosphoprotein in Normal and Cancer Tissue Slices Studied with Radio Phosphorus.*

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Experiments have been performed using radioactive isotopes to study the synthesis of phospholipids and proteins in tissue slices and homogenates.[1-6] The synthesis of large molecules, *in vitro,* can be studied by the tracer technic even though they undergo a net degradation under the conditions of the tissue slice technic.[1]

In experiments reported here, radioactive phosphorus in the form of phosphate was used to study the synthesis of total nucleic acid and phosphoprotein in both normal and tumor tissue slices, by measuring the incorporation of radioactive phosphorus, under aerobic and anaerobic conditions and in the presence and absence of added glucose. Briefly, the experiments indicate the dependence of the incorporation on the presence of oxygen, hence presumably on energy yielding reactions. The inhibition of phosphate incorporation in the absence of oxygen is partially prevented in tumor tissue, by the addition of glucose, thus suggesting that anerobic glycolysis can also provide the necessary energy.

Experimental. White rats were decapitated and the liver or kidneys removed. The slices cut were about 0.3 mm in thickness[1] and weighed about 25-30 mg when dry. They were placed in 50 cc Erlenmeyer flasks in 5 cc of Krebs-Ringer bicarbonate solution[1] containing radiophosphate. The solution had previously been equilibrated with the same gas mixture, either 95% O_2:5% CO_2 or 95% N_2:5% CO_2, as was used in the flasks during the 2 hours of incubation with shaking at 37°C. The inhibition by N_2 was determined, in each case, on slices originating from the same organ or tumor.

The concentration of radioactive phosphorus in the Krebs-Ringer bicarbonate was approximately 1 microcurie per ml. In the experiments in which glucose was added to the Krebs-Ringer bicarbonate solution, its concentration was 0.2%.

Eleven normal rats and 9 rats bearing the transplantable carcinoma 256 (Walker tumor) were utilized. Care was exercised in sampling to avoid including any of the central necrotic regions of the tumor. The body weights of the normal rats employed were about 200 g, and of the tumor bearing rats, 120 g.

* This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y., and is taken from University of Rochester Atomic Energy Report No. UR-20 (April, 1948).

† Now at Western Reserve University School of Medicine.

‡ Now at University of Texas.

[1] Taurog, A., Chaikoff, I. L., and Perlman, I., *J. Biol. Chem.*, 1942, **145**, 281.

[2] Melchior, J., and Tarver, H., *Arch. Biochem.*, 1947, **12**, 309.

[3] Winnick, T., Friedberg, F., Greenberg, D. M., *Arch. Biochem.*, 1947, **15**, 160.

[4] Frantz, I. D., Jr., Loftfield, R. B., and Miller, W. W., *Science*, 1947, **106**, 544.

[5] Frantz, I. D., Jr., Zamecnik, P. C., Reese, J. W., and Stephenson, M. L., *J. Biol. Chem.*, 1948, **174**, 773.

[6] Friedkin, M., and Lehninger, A. L., *J. Biol. Chem.*, 1949, **177**, 775.

After incubation, the nucleic acid and phosphoprotein were separated from all other phosphorus-containing substances, essentially according to the method of Schmidt and Thannhauser,[7] before counts were made. The contents of each flask were first repeatedly extracted with trichloracetic acid until the supernatant solution did not count above background, and the supernatants were discarded. In a similar manner the residue was then extracted with a mixture of alcohol and ether and finally with a mixture of chloroform and methanol; the chloroform-methanol supernatant never did count above background.

The residue, containing only total nucleic acid and phosphoprotein phosphorus, was dried, weighed, and dissolved in alkali; an aliquot of this alkaline solution was then counted. Complete solution by alkali did not uniformly occur—in some samples a small part of the residue remained finely suspended. However, since duplicates which varied in degree of solubility showed no significant discrepancy in counts, the phenomenon was ignored.

Results and discussion. The results obtained are listed in Table I. Each result is the average of 2 determinations. Each sample was counted until a total of at least 2000 counts was obtained; thus the standard error of counting in each case is less than 3%.

The results show, in the case of liver and kidney tissue, that the incorporation of radiophosphorus into nucleic acid and phosphoprotein was inhibited in nitrogen, the magnitude of the inhibition averaging about 50-70%.

The fact that no significant difference between the percentage inhibition in normal tissue as a function of the presence or absence of glucose was observed indicates that under the conditions of the experiments the absence of added substrate was not a limiting factor. In other words, perhaps more than enough substrate for anerobic glycolysis was already present in liver and kidney.

On the other hand in tumor tissue a considerable decrease in the percentage inhibition was observed when the results with

TABLE I.

Radiophosphate Uptake in Nucleic Acid and Phosphoprotein of Tissue Slices.

Tissue	Tumor			Kidney			Liver		
	% P32 uptake in O_2	% P32 uptake in N_2	% inhibition	% P32 uptake in O_2	% P32 uptake in N_2	% inhibition	% P32 uptake in O_2	% P32 uptake in N_2	% inhibition
With glucose	4.7	3.6	24	2.1	0.76	75	2.6	0.83	69
	4.0	3.4	15	2.4	0.93	62	3.2	2.1	40
	8.4	8.3	1.5				—	- -	61
	4.2	2.8	12				—	- -	32
	1.6	1.2	22						
			—			—			—
			Avg 15			Avg 69			Avg 51
Without glucose	3.1	0.37	94	1.1	0.42	60	3.1	1.7	46
	3.0	0.73	77	1.7	0.71	58	—	—	87
	2.0	0.85	59				3.6	0.85	75
	2.1	0.20	91						
			—			—			—
			Avg 80			Avg 59			Avg 69

[7] Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1945, **161**, 83.

added glucose were compared with those without added glucose. This indicates, therefore, that at least in tumor tissue under anerobic conditions and in the presence of glucose, incorporation of the major portion of the radiophosphate may depend upon glycolysis. However, the evidence presented does not permit one to rule out the possibility that the remaining radiophosphate incorporation in liver and kidney slices under anerobic conditions is an "exchange" phenomenon independent of oxidative energy.

Summary. Phosphate incorporation into nucleic acid and phosphoprotein of liver, kidney and tumor tissue slices shows some dependence upon the presence of oxygen.

The inhibition of radiophosphate incorporation into nucleic acid and phosphoprotein of tumor slices by nitrogen and its partial reversal by the addition of glucose suggest that, at least in tumor tissue, glycolysis can also serve as a source of energy for the incorporation.

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17290. Low Speed Microtomy for the Electron Microscope.

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The beam of a 50-kv electron microscope does not penetrate specimens that are thicker than 0.1 μ. Thus, in order to study biological specimens with this type of microscope we have further developed the technic described by Pease and Baker,[1] who altered a Spencer Rotary Microtome (model 820) by adding a wedge to the mechanism for forward movement. We reduced the angle of the inclined plane surface by a factor of 10 to 1 so that each step is 0.1 μ rather than 1.0 μ.

Since forward movement results from a pin in sliding contact with an inclined plane surface, it is important that the surface of the plane be as nearly perfectly flat as possible. The accompanying photograph, Fig. 1, illustrates the means by which the problem of a flat surface for even forward movement was solved. At the left of the picture (A) the original plane can be seen. The reduced angle is shown by D. The light gray triangular area (B) is an angle-reducing block of steel, to which is fastened an optical flat (C). A brass disc (E) instead of a pin is used for the feed screw tip. Horizontal movement of the disc is responsible for forward movement of the specimen. Vertical movement of the flat and the mechanism to which it is attached causes the slicing action of the microtome. Since the disc and flat are held firmly in contact by a spring, any irregularities in the surface of the plane will be evident on the sliced section.

Since the ordinary microtome knife cannot

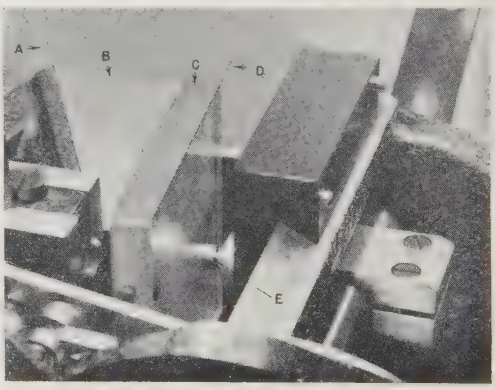

FIG. 1.
Microtome.
A. Original inclined plane surface of Spencer Microtome.
B. Steel wedge reducing from 25° to 2.5° the angle of the original plane surface with respect to direction of the feed screw movement.
C. Optical flat upon which cross feed disc rides.
D. Altered inclined plane surface, making an angle of 2.5° with direction of cross feed movement.
E. Cross feed disc.

[1] Pease, D., and Baker, R., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 470.

be used to cut sections as thin as 0.1 μ, ours was especially made,* hollow ground on both sides, with a long bevel.

Fixing and Mounting Technics. Several technics have been used to prepare tissues for microtomy. Fixation in about 4% neutral formalin, dehydration through graded alcohols, clearing in xylene, and imbedding in paraffin (85°C melting point) has proved to be one of the most successful. With this method it is not necessary to "double imbed"; that is, to use both celloidin and paraffin.

The process of mounting a tissue section on a grid for electron microscopy varies according to the specimen and the operator, but in general the following technic has been successful. The section is transferred directly from the knife to a glass slide. A dissecting needle with a microscopic point is used to lift and move the sections. The point may be inserted slightly into the edge of the paraffin of the first section of a "ribbon" and the "ribbon" pulled out somewhat. A drop of warm water added to the section on the slide will further "spread" the section. When the water has dried, a drop of xylene may be added, or the whole slide may be immersed in xylene, to dissolve the paraffin from the tissue.

When the tissue is thoroughly dry, the slide is immersed in 2% collodion in amyl acetate which is thin dried in an even film. Lines are scored around the specimen; the slide is breathed upon and immersed gently into water. The section adheres to the collodion film which strips from the glass slide and floats free. A grid is brought up beneath the specimen and both are lifted from the water. Thus the section and the supporting collodion film may be centered on the grid ready for electron observation.

Fig. 2 illustrates some results obtained with the equipment and technics described.

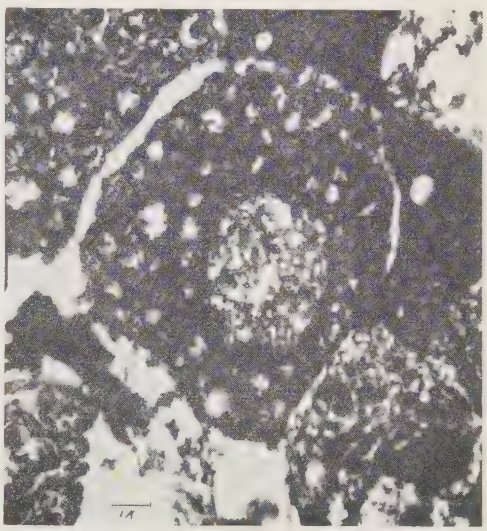

FIG. 2.
Section of rat intestine, sectioned at 0.1 μ. The greater part of the field is filled with a single cell. The nucleus appears less dense than structures seen in the cytoplasm. Individual structures have not been identified. × 13,700.

* Holzheimer, William, Melrose Park, Illinois.

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17291. The Rate and Total Loss of Body Water on the Survival Time of Adrenalectomized Frogs.*†

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One aspect of the adrenal problem is the relation of the adrenal cortex to the regulation of water and electrolytes in body fluids. The water content of certain tissues and, more pertinently, of certain cells (eviscerated carcass and liver of rats;[1] skeletal muscles of

* This investigation was aided by the Comly-Coleman Fund of the Ohio State University.

† This work was originally initiated at our suggestion by Martin W. Williams.

[1] Silvette, H., and Britton, S. W., *Am. J. Physiol.*, 1933, **104**, 399.

rats[2] and frogs,[3] and non-nucleated erythrocytes of dogs,[4] cats,[5] and rats[6]) is known to increase following adrenalectomy. Water shift in final analysis is the resultant of opposing osmotic forces acting across the plasma membrane of the particular cell in question. Since water shift, and more specifically osmotic pressure, is the variable under consideration, it appears from the very nature of the problem that an aquatic animal, like the frog, is the experimental material of choice. The frog imbibes water continuously through its integument when in an aqueous environment.[7]

The present studies were undertaken to determine the effect of known durations of exposure to a constant dehydrating force on *a,* rate and on *b,* total loss of body water, and on *c,* survival time of adrenalectomized frogs.

Method. Male frogs (*Rana pipiens*) weighing between 20 and 35 g and showing fat bodies on autopsy were used in these experiments. All frogs employed in this work may be conveniently divided into 4 groups (18-22 frogs/group): adrenalectomized frogs whose postoperative body weights were either (1) *uncontrolled,* that is, no attempt was made during the postoperative period to maintain the frog's weight at its preoperative value or (2) *controlled,* that is, the frog's postoperative body weight was maintained to within $\pm$ 1.5 g of its preoperative value, and the controls which consisted of both (3) *unoperated* and (4) *renal damaged* frogs.

Adrenalectomy was performed by "cold" cautery. Adrenal insufficiency was determined by the characteristic failure of the individual frog to perform successfully the righting reflex in not less than 3 and not more than 5 successive attempts, after previous observation of its stance and color.[8,3]

Dehydration was effected by placing the desired frog in a closed system (Scheibler desiccator) of constant volume (2230 ml). This chamber was lined, except for the upper surface and a lateral window, with a dehydrating agent (anhydrous $CaCl_2$). The system was continuously flushed during the experiment, except at the time of weighing of the frog, with washed, dried air entering via an 8-mm inlet under a pressure of 4.75 mm Hg. A given frog was placed in a closed wire basket of such size as to prevent excessive movements and of such design as to permit observation of all aspects of the body surface. This basket served both as a scale pan for it was suspended via a separate vent, provided for closure during the experiment, to a superimposed beam of an analytical balance, and as an electrode, for the basket was in circuit with 1 lead of an inductorium. The other electrode was stationary, though in such a position as to contact any portion of the ventral surface of the frog in its movable cage. A frog, after being placed in the desiccating chamber, was immediately weighed to the nearest 0.1 g. The moment of initial weight determination (elapsed time *ca.* 1 min.) was considered zero time. All subsequent weighings were made at half-hour intervals until the animal was declared dead (death point).

The death point was determined by the persistent absence of rhythmical heart beats. A very effective preliminary index, less arduous and thus less time-consuming, was the disappearance of skeletal muscle reflexes on faradic stimulation of the ventral integument with a pointed exploratory electrode, and also the less consistent, but more readily observed, buccal respiratory movements. The heart and buccal movements tend to accentuate as body volume decreases with continued dehydration. This favorable circumstance tends to offset the enfeebling of these movements prior to the terminal stage.

Autopsies were performed on all frogs immediately upon termination of an experimental run in order to confirm the continued

[2] Crismon, J. M., and Field, J., 2nd., *Am. J. Physiol.,* 1940, **130**, 231.

[3] Angerer, C. A., and Angerer, H. H., *Fed. Proc.,* 1942, **1**, 3.

[4] Harrop, G. A., *Bull. Johns Hopkins Hosp.,* 1936, **59**, 11.

[5] Hegnauer, A. H., and Robinson, E. J., *J. Biol. Chem.,* 1936, **116**, 769.

[6] Gonzalez Q., J., and Angerer, C. A., *Am. J. Physiol.,* 1947, **149**, 502.

[7] Adolph, E. F., Physiological Regulations, p. 110, The Jaques Cattell Press, Lancaster, Pa., 1943.

[8] Maes, J., *Arch. Intern. de Physiol.,* 1937, **45**, 135.

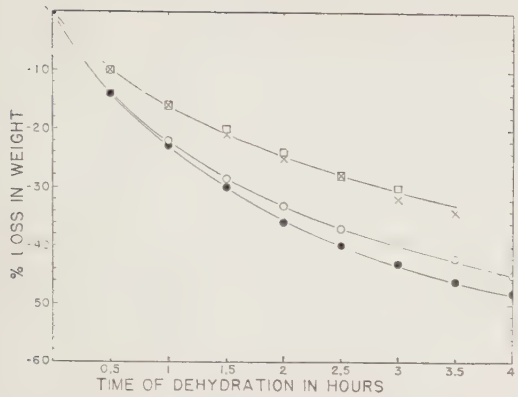

FIG. 1.

Percentage loss in body weights (water loss) of 4 groups of frogs plotted as functions of respective times (readings at ½ hour intervals) of exposure to a constant force of dehydration. The characters used to delineate the curves for each group are as follows: adrenalectomized frogs whose postoperative body weights were either uncontrolled (X), or controlled (□) to within ± 1.5 g of their respective preoperative values, and the control frogs—renal damaged (○) and unoperated (●). Volume of dehydrating system = 2230 ml. dry air current + 4.75 mm Hg, temp. 18-22°C.

absence of heart action and presence of fat bodies. In only 3 frogs of the 4 groups studied were feeble heart beats observed on autopsy which were not detected immediately before. These hearts failed to survive the half-hour interval following the close of the experimental run. These data are not included in the results presented.

Results. The various mean values for the percentage loss in body weights for the 4 groups of frogs are plotted in Fig. 1 as functions of time of exposure in hours to a constant dehydrating force. The means of the various data have been statistically compared for significance by "Student's" method and are presented together with their respective "*t*" values in Table I. The figure and table are self-explanatory after recourse to their accompanying legends.

When data from the 4 groups of frogs are analyzed for *a,* rate of loss and *b,* total loss of body weight, and *c,* duration of survival, the following orders of statistical significance are indicated (see Table I): the 2 groups of controls (renal damaged and unoperated) show no significance for the foregoing items *b* and *c,* though *a* is probably significant. For reasons to be discussed, the renal damaged frogs are considered the controls for all subsequent comparisons. A comparison of data from the adrenalectomized frogs gives no significance for the aforementioned items *a, b,* and *c.* However, when either group of adrenalectomized frogs are compared with their controls, a high degree of significance is found for items *a* and *b,* and for item *c* as it affects the controlled but not the uncontrolled adrenalectomized frogs.

Discussion. The assumption is made here that any loss in body weight during the relatively short period of exposure to a constant force of dehydration is due to loss of body water.

The only significant difference between data from the 2 groups of controls (unoperated and renal damaged) lies in the respective rates of water loss. This difference is interpreted as due to the reduction in the total effective dehydrating surface of operated frogs arising from the encroachment of the thickened integument at the line of suture (4-5 cm) and of the loss in integument in effecting this suture. For this reason, the renal damaged frogs must be regarded as the true controls.

Since the difference between the respective means for data obtained from the 2 adrenalectomized groups is not significant, it remains to compare both of these groups with their controls. It has been shown that uncontrolled adrenalectomized frogs undergo postoperatively a progressive increase in body weight, so that, *e.g.,* on 7 and 12 days the mean body weight has increased by 28 and 33% respectively.[9] The 2 groups of adrenalectomized frogs when compared with the control show a highly significant decrease in the rate and in the total loss of body water; they differ between themselves in that the survival time is probably significant ($P < 0.02$) for the controlled but not for the uncontrolled ($P > 0.05$) adrenalectomized frogs. Were, possibly, the increase in osmotic pressure, resulting from forced dehydration, the underlying cause of this difference, then the uncontrolled, having the greater initial water load

[9] Angerer, C. A., unpublished data.

TABLE I.
Summary and Comparison (t-test) of Mean Values for All Data (Line 3) Obtained from the Various Groups of Frogs Subjected to a Constant Force of Dehydration.

Frogs		Adrenalectomized						Control					
Experimental groups		1 Wt uncontrolled			2 Wt controlled			3 Normal			4 Renal damaged		
Statistical classes		*a* %	*b* %	*c* hr	*a* %	*b* %	*c* hr	*a* %	*b* %	*c* hr	*a* %	*b* %	*c* hr
Mean		24.2	32.2	3.3	24.6	28.4	2.7	35.8	48.0	4.0	32.9	43.4	3.8
S.E. ±		0.7	1.9	0.3	1.2	1.4	0.4	1.0	1.0	0.1	0.7	1.2	0.1
S.D. ±		2.3	6.6	1.0	4.0	4.7	1.4	3.4	3.5	0.4	2.3	4.0	0.5
t-test	*2*	0.30 NS	1.63 NS	1.19 NS									
	4	9.32 HS	5.07 HS	1.55 NS	6.20 HS	8.43 HS	2.54 S	2.46 S	1.53 NS	1.09 NS			

Any given letter among the following (line 3) indicates the same variable studied in any group of frogs (lines 1 and 2) and this meaning is used throughout the text:
a = % loss of body weight at the end of 2-hour period of dehydration;
b = % total loss of body weight at death-point;
c = Duration of dehydration in hours until death-point.
S.E. = Standard error, and S.D. = Standard deviation of respective mean.
t-test = Statistical comparison of ratio of difference between an indicated pair of means/estimated standard error of this difference.
Statistically: NS = Not significant (P >0.05); S = Significant (P 0.05-0.01); HS = Highly significant (P <0.01).

in comparison with the controlled adrenalectomized frogs, not only should survive longer but also should suffer the greater rate and the greater total loss of body water. None of these postulates is met on comparing the 2 groups of adrenalectomized frogs; but on comparing the latter groups with their controls the reverse tends to be true. The conclusion reached is that the adrenalectomized frogs can tolerate relatively slight loss in body water before lethal effects are encountered. This is not due to any decrease in permeability of the integument of adrenalectomized frogs, for all evidence points to an increase in permeability[10] in general and for frog skin[9] in particular.

The decreased rate of dehydration found in both groups of adrenalectomized frogs may be interpreted as due to the immediate decrease in replenishment of body fluid at the body surface. This condition may arise from the hemoconcentration and hemostasis known to occur in peripheral vessels as a result of adrenocortical insufficiency or ablation.[11] Physiologically, forced dehydration has much in common with sweating but without benefit of the concomitant vasodilatation arising from the increase in environmental temperature. Sweating, and dehydration, like adrenocortical insufficiency, leads to a further decrease in plasma volume.[12] Thus, a decreased plasma volume, together with an increased blood viscosity and osmotic pressure, has a deleterious effect on an already weakened heart action[13] which follows in the wake of adrenalectomy.

That mammals deficient in adrenal cortical hormone are less able to withstand various forms of stress (certain types of drugs, poisons, toxins, infections, variations in environmental temperature, barometric pressure, and traumatic procedures) is too well-known to require elaboration.[14] Thus, an increase in osmotic pressure induced either by a re-

[10] Hartman, F. A., *Endocrinology*, 1942, **30**, 861.

[11] Swingle, W. W., Vars, H. M., and Parkins, W. M., *Am. J. Physiol.*, 1934, **109**, 488.

[12] Adolph, E. F., Physiology of Man in the Desert, p. 170, Interscience Publishers, Inc., New York, N. Y., 1947.

[13] Nicholson, W. M., and Soffer, L. J., *Bull. Johns Hopkins Hosp.*, 1935, **56**, 236.

[14] Swingle, W. W., and Remington, J. W., *Physiol. Rev.*, 1944, **24**, 89.

duction of free-moving particles or by a possible increase of osmotically-active particles, is to be considered as another example of stress with which the adrenalectomized organism fails to cope.

Summary. All frogs employed in this study may be conveniently divided into 4 groups (18-22 animals group): the adrenalectomized frogs whose postoperative body weights were either controlled, to within ± 1.5 g of their individual pre-operative values, or uncontrolled; and the controls, both renal damaged and unoperated frogs. The individuals of each group were subjected to a constant dehydrating force and the resulting data were statistically analyzed with respect to the following points: *a*, the rate of loss and *b*, the total loss of body weight (water) and *c*, the duration of survival on exposure to a constant force of dehydration.

1. A comparison of the difference between respective means of the 2 groups of adrenalectomized frogs shows no significance as regards the foregoing items *a*, *b*, and *c*. 2. Comparison between the 2 groups of controls (renal damaged and unoperated frogs) gives no significance with respect to items *b* and *c*, though it does for *a*. 3. A comparison between the respective means for either group of adrenalectomized frogs and their controls (renal damaged) produces a significance for *a* and *b*, and for *c* as it affects the death point of the controlled but not of the uncontrolled adrenalectomized frogs. 4. On the basis of the known cardiovascular embarrassment subsequent to adrenalectomy, it is suggested that the increased osmotic pressure resulting from the forced water loss and the attendant decrease in peripheral circulation brings an increased osmotic stress to bear on an already weakened heart action. It is suggested that this stress is the deleterious factor in affecting the physiological points raised.

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17292. Use of Antitryptic Agents in Tissue Culture. I. Crude Soybean Trypsin-Inhibitor.*†

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Substances that inhibit the proteolytic activity of trypsin have been found in serum and plasma,[1] in egg white,[2] in navy beans and soybeans,[3] and in extracts of pancreas.[4,5] Because of the great activity of these antitryptic agents, it seemed of interest to investigate the possibility of using them in tissue culture as a means of preventing the digestion of the plasma coagulum that frequently occurs during the growth of cells *in vitro*.[6-8] The soybean antitrypsin,‡ which has been

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† Grateful acknowledgement is made to Miss M. Ogilvie, Mrs. C. J. Porter, and Mr. C. J. MacFayden for technical assistance.

1 Grob, D., *J. Gen. Physiol.*, 1943, **26**, 405.

2 Balls, A. K., and Swenson, T. L., *J. Biol. Chem.*, 1934, **106**, 409.

3 Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 139.

4 Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

5 Kazal, L. A., Spicer, D. S., and Brahinsky, R. A., *J. Am. Chem. Soc.*, 1948, **70**, 3034.

6 Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, **13**, 495.

7 Losee, J. R., and Ebeling, A. H., *J. Exp. Med.*, 1914, **19**, 593.

8 Santesson, L., *Acta path. et microbiol. Scand.*, 1935, Suppl. **24**

‡ It is interesting to note that Fischer has just reported (Fischer, A., *Science*, 1949, **109**, 611) a series of experiments with crystalline soybean trypsin inhibitor supplied by Kunitz.[9] His results are in complete accord with those reported here.

9 Kunitz, M., *J. Gen. Physiol.*, 1947, **30**, 291.

shown by Kunitz[9] to be a protein of globulin nature, was selected as the first agent to be studied in the present investigation.

Methods. Crude trypsin-inhibitor was prepared from solvent-extracted unheated soybean flakes§ by the method of Kunitz.[10] This material was dried from the frozen state, and the resulting powder was kept in the refrigerator. The activity of each preparation was determined by its ability to prevent the digestion of casein by trypsin, according to the procedure of Kunitz.[9] In these tests, graded amounts of antitrypsin were added to a standard solution of trypsin and the degree of proteolysis was then measured colorimetrically as tyrosine liberated by Folin's reagent. For use in tissue cultures, the dried powder was dissolved in 0.01 N HCl, and the material was sterilized by passage through a UF fritted glass filter. Stock solutions were diluted further in sterile glass-distilled water to the desired concentrations. All sterile solutions were kept in the refrigerator, and were found to retain their antitryptic potency for a period of at least 6 to 8 months.

Culture strains of fibroblast-like cells were derived from the leg muscle of 11-day chick embryos, and were cultivated in D-3.5 flasks through at least 10 to 12 passages (weeks) before use. In some experiments, use was made of fresh tissue explants.

The coagulum added to each flask consisted of 0.3 ml chicken plasma, 0.3 ml chick embryo extract, 0.3 ml Earle's modification of Tyrode's solution,[11] and 0.1 ml phenol red (0.025% in Earle's solution). In addition, a feeding mixture was added to each culture flask on the second day and was renewed on the fourth and sixth days. This feeding mixture was comprised of 0.3 ml embryo extract, 0.6 ml Earle's solution, and 0.1 ml phenol red solution. At no time was the original coagulum reinforced by the addition of another layer of plasma.

§ Obtained through the courtesy of Dr. W. D. McFarlane, Canadian Breweries, Ltd., Toronto, Ontario.

[10] Kunitz, M.; *J. Gen. Physiol.*, 1946, **29**, 149.

[11] Earle, W. R., *J. Nat. Cancer Inst.*, 1943, **4**, 165.

Embryo extract was prepared by grinding 11-day chick embryos that were suspended in a roughly equivalent volume of Earle's solution in a graduated all-glass homogenizer. After the cell debris had been separated by centrifuging, the supernatant was frozen and thawed twice. This concentrated extract was diluted in Earle's solution to 1 part in 4 and was kept as a stock solution in the refrigerator. The stock solution was further diluted in Earle's solution to a final concentration of 1 part in 20 immediately before use.

The antitryptic factor (0.1 ml of an appropriate dilution) was added to both the original coagulum and the feeding mixture in place of 0.1 ml of Earle's solution. An equal volume of glass-distilled water was added to all control cultures.

In some experiments, the effect of antitrypsin was tested on cell colonies cultivated in fibrinogen-thrombin clots prepared by the method of Porter and Hawn.[12] To these cultures, 0.3 ml of serum was added to provide the nutrient materials ordinarily supplied by the plasma.

When the tissue transplants were prepared from strain material, each of the cultures chosen for an experiment was cut into 4 fragments of approximately equal size, and 3 of the fragments were embedded in separate flasks containing graded amounts of antitrypsin. The fourth fragment in each set served as a control and was cultivated in the absence of antitrypsin. Each experiment consisted of at least 3 sets of cultures containing the same ingredients.

To test for possible inhibition of migration and growth by the antitryptic agent, outline drawings showing the increase in surface area were made daily by means of a projectoscope, and the increments were measured with a planimeter.

Results. Prevention of clot digestion. In preliminary experiments, crude antitrypsin, prepared as described, was added to a series of 12th passage sister cultures at final concentrations of 5.0, 3.0, 2.0, 1.0, 0.5, 0.25, 0.1, and 0.05 mg per ml. These cultures together with

[12] Porter, K. R., and Hawn, C. v. Z., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 309.

their controls were incubated for 7 days and were observed each day for signs of digestion of the coagulum. It was found that antitrypsin levels between 5.0 mg per ml and 0.25 mg per ml completely prevented digestion of the plasma coagulum, while lower concentrations were not effective. Figs. 1 and 2 show two representative cultures selected from this series and photographed at 11 days.

Comparable results were also obtained in other experiments in which fibrinogen-thrombin clots were used. In these instances, the effective concentrations of antitrypsin were found to be between 5.0 and 0.25 mg per ml.

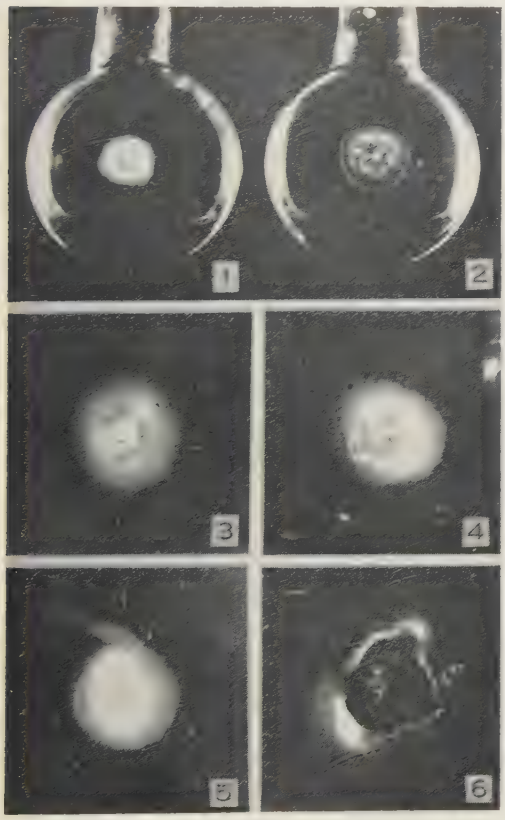

Fig. 1 and 2.

Eleven-day sister cultures of 12th passage chick fibroblasts cultivated in plasma medium in the presence of 0.5 mg antitrypsin per ml (Fig. 1), and in the absence of antitrypsin (Fig. 2). × 1.

Fig. 3, 4, 5 and 6.

Seven-day sister cultures of 15th passage chick fibroblasts cultivated in plasma medium in the presence of 1.0 mg antitrypsin per ml (Fig. 3), 0.5 mg per ml (Fig. 4), 0.25 mg per ml (Fig. 5), and in the absence of antitrypsin (Fig. 6). × 3.

In both types of coagulum, control cultures usually gave evidence of initial digestion within 24 to 48 hours. This appeared first as a thin ring in the medium a short distance from the tissue fragment, and progressed rapidly to form a crater containing liquefied plasma. The tissue fragment contracted very considerably, and in most cases was found floating freely in the liquid plasma. Cell growth frequently occurred on the surface of the glass beneath the digested area.

Inhibition of plasma coagulation by antitrypsin. It was found that the presence of high concentrations of the crude trypsin-inhibitor (5.0 to 1.0 mg per ml) prevented normal plasma coagulation in the presence of embryo extract. The addition of a few drops of dilute thrombin overcame this inhibition and permitted normal clot formation. This finding is in agreement with the results of Tagnon and Soulier,[13] who reported that both crude and crystalline antitrypsin from soybeans had a marked anticoagulant effect upon whole blood and recalcified plasma, but had no antithrombic activity. In the present investigation, it was also noted that the anticlotting activity of trypsin-inhibitor was more pronounced when heparin or citrate was present in the plasma.

Inhibition of tissue growth by antitrypsin. The presence of crude soybean antitrypsin was found to retard the growth of the culture strains that were used as test material. The effect was most pronounced at high concentrations, 3.0 to 1.0 mg per ml. Cultures maintained in such high antitrypsin concentrations were observed to have a total area that was 20 to 30% less than the area of sister cultures grown in the absence of antitrypsin. The toxicity was less evident at lower levels, 0.5 to 0.25 mg per ml. Inhibition of growth was not uniform, however, in all cultures tested; and in some experiments the inhibition was negligible even at concentrations of 3.0 mg per ml. The effect of graded levels of crude antitrypsin on 15th passage strain cultures is shown in Fig. 3, 4, 5 and 6.

Repeated passage of cultures through media

[13] Tagnon, H. J., and Soulier, J. P., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 440.

containing inhibitory levels of antitrypsin resulted in a gradual disappearance of the growth-depressing effect. The cultures acquired a tolerance to antitrypsin after 3 to 5 passages, and thereafter appeared to grow normally in the presence of concentrations that had previously been found to be inhibitory.

Effect of trypsin-inhibitor on fresh tissue explants. When a study was made of the effect of several concentrations of crude soybean antitrypsin on fresh tissue explants in their first culture passage, it was found that levels of 1.0, 0.5, and 0.25 mg per ml completely prevented digestion of the plasma coagulum. It was also noted that fresh tissues were much less sensitive to the toxic action of antitrypsin than were strain cultures, and concentrations as high as 3.0 mg per ml had no appreciable inhibitory effect. This absence of growth inhibition was also observed in cultures of fresh tissues carried in fibrinogen-thrombin clots.

Discussion. Digestion of the plasma coagulum by growing cells has presented a considerable problem since the earliest days of tissue culture. Thus, Losee and Ebeling,[7] working with human culture material, attempted to prevent liquefaction of the plasma by the addition of serum, agar and egg albumin. Although these experiments were unsuccessful, it was found that dilution of the plasma with an equal volume of Ringer's solution slowed down the rate of digestion sufficiently to allow short term experiments to be carried out. Carrel[14] reported that he was able to protect fibrin clots from digestion by the addition of a little serum, a small amount of sodium linoleate, or a suspension of egg yolk. Of the various sera that have been employed, horse serum has proved to be most suitable, although it has the undesirable property of retarding the rate of cell multiplication. In many laboratories, the proteolytic action of tissues cultivated in plasma is controlled by the addition of a second layer of plasma, on the day following preparation of the cultures. Subsequent layers of plasma are then added as they become necessary. This procedure takes considerable time and requires large amounts of plasma.

Some investigators[6,14] have attributed digestion of the plasma coagulum to proteolytic enzymes secreted by the cells during active growth. Others[15–17] have advanced the hypothesis that cells in tissue culture elaborate an activating substance that transforms an inactive precursor, profibrinolysin, present in the medium, into an active proteolytic enzyme, fibrinolysin, which then digests the clot. The present observations can be interpreted on the basis of either of these hypotheses.

The results reported in the present paper indicate that the incorporation of antitryptic agents in the culture medium offers promise of a solution to the troublesome problem of clot digestion. It is realized, of course, that the slight inhibition of growth caused by crude soybean antitrypsin presents a serious obstacle to its routine use. But further studies are now in progress with more highly purified preparations of the trypsin-inhibitor in an effort to eliminate the growth-retarding factor. It is also planned to extend the present investigation to a study of antitryptic agents derived from other sources.

Summary. Crude soybean trypsin-inhibitor has been found to prevent digestion of plasma and fibrinogen-thrombin clots by tissues growing *in vitro*. The presence of antitrypsin retards the normal coagulation of plasma but this inhibition can be overcome by the addition of thrombin. The growth of strain cultures of fibroblast-like cells is somewhat depressed by high concentrations of antitrypsin, but the inhibitory effect can be eliminated by repeated cultivation in antitrypsin. Fresh tissue explants are not as sensitive as strain cultures to this inhibitory action.

[14] Carrel, A., *J. Exp. Med.*, 1923, **38**, 407.

[15] Demuth, F., and von Riesen, I., *Biochem. Z.*, 1928, **203**, 22.

[16] Astrup, T., and Permin, P. M., *Nature*, 1947, **159**, 681.

[17] Goldhaber, P., Cornman, I., and Ormsbee, R. A., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 590.

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17293. Studies of Experimental Pulmonary Edema. I. Pulmonary Edema from *l*-Epinephrine and *l-nor*-Epinephrine (Arterenol).*

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Large doses of epinephrine result in death, accompanied by an acute pulmonary edema in various species.[1-3] Studies of pulmonary edema caused by a number of compounds suggested that the mechanism may be by way of the release of pressor amines from the adrenal medulla. The recent discovery that this organ contains[4-9] and releases *l-nor*-epinephrine[10] as well as *l*-epinephrine made it desirable to compare the production of pulmonary edema by these compounds.

Experimental. Unfasted guinea pigs were used with equal sex distribution in the various groups. The *l*-epinephrine† and the *l-nor*-epinephrine (*l*-arterenol) were administered as the hydrochlorides in 0.9% NaCl in concentrations near 0.1%. In those experiments in which it was used, the adrenergic blocking agent, N-(9-fluorenyl)-N-ethyl-β-chlorethylamine-HCl ("SKF-501")‡ was dissolved in saline as a 0.1% solution for doses of 2 mg/kg 10-15 minutes before the pressor amine. The solutions were given intravenously via the penile vein in males and intracardially in female animals. There were 6 guinea pigs in each group. The degree of pulmonary edema was determined by the weight of the lungs.[3]

Results. Our data are summarized in Table I. The *l*-epinephrine, except in the dose of .001 mM/kg (from which they recovered and were sacrificed with ether an hour later), was rapidly fatal. Groups 3 and 4 survived an average of 11 and 4 minutes respectively. On the basis of previous experience, *l-nor*-epinephrine was used in larger doses than the epinephrine but although the higher doses (Groups 8 and 9) prostrated the animals, they recovered and were etherized an hour after the drug was given. Higher doses of *nor*-epinephrine were required to cause pulmonary edema and with the highest dosage used, .004 mM/kg, it failed to exceed that which resulted from one-fourth as much epinephrine.

These results are in accord with those of Tainter, Tullar and Luduena[11] who found that the LD_{50} of intravenously administered *l-nor*-epinephrine in mice is eight times less than that for *l*-epinephrine. Undoubtedly these results were in part due to pulmonary edema.

Stone and Loew,[3] and others have shown that certain adrenergic blocking agents are capable of reducing epinephrine-induced pulmonary edema in animals and this effect has been used as one measure of the effectiveness of adrenergic blocking agents. We administered N-(9-fluorenyl)-N-ethyl-β-chlorethylamine ("SKF-501"), a β-haloalkylamine, 10-15 minutes before the pressor amines. It prevented the occurrence of any symptoms whatever or of grossly measurable pulmonary edema otherwise induced by toxic doses of either *l*-epinephrine or *l-nor*-epinephrine.

* Supported by a grant from the Life Insurance Medical Research Fund.

1 Meltzer, S. J., *Am. Med.*, 1904, **8**, 191.

2 Emerson, H., *Arch. Int. Med.*, 1909, **3**, 368.

3 Stone, C. A., and Loew, E. R., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 122.

4 Schümann, H., *Klin. Wschr.*, 1948, **26**, 37.

5 Holton, P., *Nature*, 1949, **163**, 217.

6 Pitcairn, D. M., and Youmans, W. B., *Fed. Proc.*, 1949, **8**, 127.

7 Euler, U. S. von, and Hamberg, U., *Nature*, 1949, **163**, 642.

8 Goldenberg, M., Faber, M., Alston, E. J., and Chargaff, E. C., *Science*, 1949, **109**, 534.

9 Tullar, B. F., *Science*, 1949, **109**, 536.

10 Bulbring, E., and Burn, J. H., *Nature*, 1949, **163**, 363.

† The *l*-epinephrine (Adrenalin) used was a specially purified sample of the natural product for which we are indebted to Dr. Leon A. Sweet of Parke, Davis & Company. It contained only a trace of *l-nor*-epinephrine (arterenol).

‡ We are indebted to Dr. Glen E. Ullyot of the Smith, Kline & French Laboratories for generous supplies of "SKF-501."

11 Tainter, M. L., Tullar, B. F., and Luduena, F. P., *Science*, 1948, **107**, 39.

TABLE I.
Pulmonary Edema Caused by *l*-epinephrine and *l-nor*-epinephrine in Guinea Pigs.

Group No.	Dose of drug, mM/kg body wt*	Avg body wt, g	Avg lung wt g/100 g body wt	% change
Controls				
1	0	540	0.55 ± .09	—
l-epinephrine				
2	.0010	585	1.27 ± .14	+131
3	.0015	520	1.72 ± .13	+210
4	.0020	615	1.62 ± .06	+191
l-epinephrine preceded by N-(9-fluorenyl)-N-ethyl-β-chlorethylamine HCl†				
5	.0020	605	0.55 ± .08	0
6	.0050	630	0.56 ± .08	+ 2
l-nor-epinephrine (*l*-arterenol)				
7	.0020	515	0.97 ± .16	+ 76
8	.0030	480	1.13 ± .18	+105
9	.0040	505	1.25 ± .26	+127
l-nor-epinephrine preceded by N-(9-fluorenyl)-N-ethyl-β-chlorethylamine HCl†				
10	.0040	550	0.51 ± .12	— 7
11	.0050	510	0.58 ± .11	+ 5

* 1 mM epinephrine = 183 mg and 1 mM *nor*-epinephrine = 169 mg. † Adrenergic blocking agent known as "SKF-501" (see text).

Summary. 1. As measured by lung weight a lesser degree of pulmonary edema is produced by toxic doses of *l-nor*-epinephrine than smaller but toxic doses of *l*-epinephrine.

2. N-(9-fluorenyl)-N-ethyl-β-chlorethylamine, a β-haloalkylamine adrenergic blocking agent, prevents all symptoms and the development of pulmonary edema due to toxic doses of either *l*-epinephrine or *l-nor*-epinephrine.

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17294. The Mechanism by Which Dibenamine Blocks Pituitary Activation in the Rabbit and Rat.

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(Introduced by Duncan C. Hetherington.)
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Markee, Sawyer, and Hollinshead[1,2] presented strongly indicative evidence that hypothalamic control of the release of luteinizing hormone from the rabbit hypophysis is exerted via a neurohumoral mechanism of which an adrenergic mediator is the final component. Ovulation, significant of LH release, was induced by injecting tiny amounts of adrenalin directly into the adenohypophysis.[2] Sawyer *et al.*[3,4] reported confirmation of the adrenergic nature of the secretion stimulus by blocking copulation-induced ovulation with the adrenergic-blocking agent dibenamine (N,N-dibenzyl-β-chloroethylamine).[5] Everett, Sawyer, and Markee[6,7] extended the investigation

1 Markee, J. E., Sawyer, C. H., and Hollinshead, W. H., *Endocrinology*, 1946, **38**, 345.

2 Markee, J. E., Sawyer, C. H., and Hollinshead, W. H., *Recent Progr. Hormone Research*, 1948, **2**, 117.

3 Sawyer, C. H., Markee, J. E., and Hollinshead, W. H., *Endocrinology*, 1947, **41**, 395.

4 Sawyer, C. H., Markee, J. E., and Townsend, B. F., *Endocrinology*, 1949, **44**, 18.

5 Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1948, **7**, 397.

TABLE I.
Effects of Dibenamine, Priscol and 2-Dibenzylaminoethanol on Activation of LH-Release by the Anterior Hypophysis.

Species	Drug	Dose, mg/kg	Time of injection (relative to nervous stimulus)	Ovulated (hypophysis stimulated)	Failed to ovulate (stimulus blocked from reaching hypophysis)	% blocked by drug
Rabbit	Dibenamine*	25-32	<60 sec. after	3	16	84
	Priscol*	40-52	<60 sec. after	9	1	10
	2-dibenzyl-aminoethanol	25-30	<45 sec. after	9	1	10
Rat	Dibenamine†	30	0-6 hr before	8	21	72
	2-dibenzyl-aminoethanol	30	>2 hr before	5	0	0

* Data from Sawyer *et al.*[3]
† Data from Everett *et al.*[7] Six of the 8 ovulating rats were partially "blocked" as evidenced by incomplete or delayed ovulation.

to the rat, finding that, in this spontaneously ovulating species, dibenamine also blocked LH-release.

Dibenamine effectively blocked ovulation in the rabbit only if injected intravenously within 60 seconds post coitum; if delayed a few minutes, ovulation followed in all cases.[3] Since dibenamine does not exert its maximal adrenergic-blockade until at least 1.5 hours after injection, Nickerson[8] recently suggested that the drug may inhibit LH release in the rabbit by a central nervous effect independent of adrenolytic activity. Nickerson suggested further that this possibility be controlled by the use of 2-dibenzylaminoethanol, a dibenamine-hydrolysis product which retains the central excitant properties but lacks the adrenergic-blocking power of dibenamine. The present experiments demonstrate that 2-dibenzylaminoethanol does not prevent activation of LH-release in the rabbit or rat, and that at least certain anti-adrenergic properties of dibenamine are exerted within a matter of seconds after intravenous administration.

Sexually mature female rabbits and rats were employed. The rabbits were mated with a vasectomized buck, and 2-dibenzylaminoethanol* was injected intravenously during the following 45 seconds. The rats, whose vaginal-smear records had each shown two or more regular 4-day cycles, were injected with the drug intravenously at noon on the day of proestrus. Previous work[7] had revealed that 4-day cyclic rats of our colony receive the neurogenic stimulus for the release of LH between 2 and 4 P.M. on the afternoon of proestrus.

The central excitatory effects of 2-dibenzylaminoethanol in rabbits are more dramatic though shorter lived than those induced by dibenamine. The most commonly employed dosage of 2-dibenzylaminoethanol, 25 mg/kg, always induced severe convulsions lasting up to 20 minutes, and this dose level was nearer the LD_{50} than was 30 mg/kg dibenamine. A dosage of 30 mg/kg 2-dibenzylaminoethanol was above the MLD for rats, but in this species the excitatory effects were somewhat less marked than those following a similar dose of dibenamine.

The results of injections of 2-dibenzylaminoethanol, as they pertain to pituitary activation are summarized in Table I and compared with the results of earlier work with dibenamine and priscol (benzyl-imidazoline).

[6] Sawyer, C. H., Everett, J. W., and Markee, J. E., *Endocrinology*, 1949, **44**, 218.

[7] Everett, J. W., Sawyer, C. H., and Markee, J. E., *Endocrinology*, 1949, **44**, 234.

[8] Nickerson, M., *Endocrinology*, 1949, **44**, 287.

* Generously supplied by Dr. Nickerson.

TABLE II.
Relative Potencies of Drugs to Counteract Immediately a 2X-Lethal Dose of Adrenalin in Rabbits.

Drug	Dose, mg/kg	Timing of drug injection in seconds from 0 time: Began	Timing of drug injection in seconds from 0 time: Ended	Timing of 1 mg/kg adrenalin inj. in seconds from 0 time: Began	Timing of 1 mg/kg adrenalin inj. in seconds from 0 time: Ended	Survived	Died	% protection
—	—	—	—	0	15	0	10	0
Dibenamine	30	0	45	45	60	5	0	100
Priscol	40	0	45	45	60	1	4	20
2-Dibenzyl-aminoethanol	25	0	45	45	60	0	3	0

It is apparent that, whereas dibenamine is highly effective in blocking pituitary activation in both rabbits and rats, 2-dibenzylaminoethanol, like priscol, is quite ineffective in preventing the neurogenic stimulus from reaching the hypophysis.

A second series of experiments was designed to demonstrate whether dibenamine exerts any of its anti-adrenergic activities within the first minute following intravenous injection. The experiment is outlined and the results summarized in Table II. The rabbits receiving the intravenous adrenalin alone immediately revealed maximally dilated pupils, muscular hypotonia and respiratory embarrassment followed in a few minutes by death. When adrenalin injections were preceded within the minute by dibenamine, the subsequent symptoms were those of dibenamine only—pupillary constriction and spontaneous muscle contractions. Priscol delayed the lethal effect of adrenalin for many hours in 4 animals, and a fifth recovered. However, neither priscol nor 2-dibenzylaminoethanol counteracted the pupillary dilation induced by adrenalin. The 3 rabbits receiving 2-dibenzylaminoethanol and adrenalin expired as rapidly as with adrenalin alone. Although protection against the lethal action of adrenalin is not a highly specific test of adrenolytic activity,[9] the fact that dibenamine-treated rabbits showed none of the symptoms of adrenalin poisoning mentioned above, indicates that at least part of dibenamine's adrenergic-blocking capacity becomes effective almost immediately after injection of high dosages. The results indicate that dibenamine blocks pituitary activation, not by its central excitatory effects but rather by its specific adrenergic-blocking capacity.

Summary. Dibenzylaminoethanol, a non-adrenolytic dibenamine-hydrolysis product which retains the central excitatory action of dibenamine, fails to block the activation of LH release from the rabbit or rat hypophysis. Dibenamine in large doses exerts at least part of its anti-adrenergic properties almost immediately after injection. The evidence indicates that dibenamine blocks pituitary activation by its specific adrenergic-blocking capacity rather than by its central excitatory action.

[9] Nickerson, M., Berghout, J., and Hammerstrom, R. N., *Fed. Proc.*, 1949, **8**, 322.

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17295. Intraventricular Pressure Curves of the Human Heart Obtained by Direct Transthoracic Puncture.*

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In 1943, one of us (W.C.B.) studying blood pressures by the direct puncture technic had occasion to obtain a pressure curve from both the right and left ventricle by passing a needle directly into the cavity of these heart chambers. The recent development of right heart catheterization and the attempt to catheterize the left ventricle in man via the carotid artery, a procedure not without hazard, suggested to us the merits of publishing this experience with direct heart puncture. This is particularly timely because the experience with catheterization has shown not only how difficult but also how important for proper diagnosis it is to obtain adequate pressure curves. This difficulty obviously could be avoided by using the direct puncture technic as opposed to the long catheter method. Unfortunately, the hazards associated with direct puncture rule out this method for ordinary use.

The record shown in Fig. 1 was obtained on a 65 year old male who was moribund because of a cerebral thrombosis and terminal bronchopneumonia. The procedure had no significant effect upon the clinical course. A Hamilton needle manometer of adequate frequency similar to that usually employed for intra-arterial pressure recordings was used to record the intracardiac pressures. This was mounted on a portable electrocardiographic machine as previously described.[1] The right ventricular pressure curve (Fig. 1) was obtained by inserting the needle in the fourth intercostal space just to the left of the sternum. No electrocardiogram was recorded at this time. A respiratory fluctuation in the right ventricular pressure curve can be seen, the minimum end diastolic presure was 15 mm Hg and the maximum systolic pressure was 40 mm Hg. There was thus evidence of right heart congestion with little increase in systolic pressure. The contour of the curve is very satisfactory and shows none of the distorting artifacts so commonly seen with the catheter technic.

The top segment shows lead 2 of the electrocardiogram together with the left ventricular pressure curve. This was obtained by inserting the needle in the fifth intercostal space just beyond the left nipple line where the maximum apex beat was felt. In this instance there are frequent ventricular premature systoles occurring at times as pairs and triads. The third beat after the first pair of ventricular premature systoles is a fusion beat (labelled 3 on the left ventricular pressure curve). The beginning and end of the record present an acceptable undistorted left ventricular pressure curve. The pressure in this ventricle varied with respiration, showed a minimal end diastolic pressure of 15 mm Hg and a maximum systolic pressure of 100 mm Hg.

In both the left and right ventricular pressure curves, a noticeable auricular pressure wave is seen to precede the ventricular curve and is responsible to a large extent, especially in the left side, for the high diastolic end pressure. The slope of the ventricular pressure curves throughout systole and diastole is similar to that obtained by adequate pressure recordings from the cavities of the dog's heart.

The middle section of the top record offered the opportunity of studying the effect of premature systoles upon the pressure curve of the left ventricle. The beats of interest in the *middle section* have been indicated by numbers from 1 to 10. They will be referred to by these numbers in the subsequent discussion.

† This department is supported, in part, by the Michael Reese Research Foundation.

* Aided by the A. D. Nast Fund.

1 Buchbinder, W. C., and Sugarman, H., *Arch. Int. Med.*, 1940, **66**, 625.

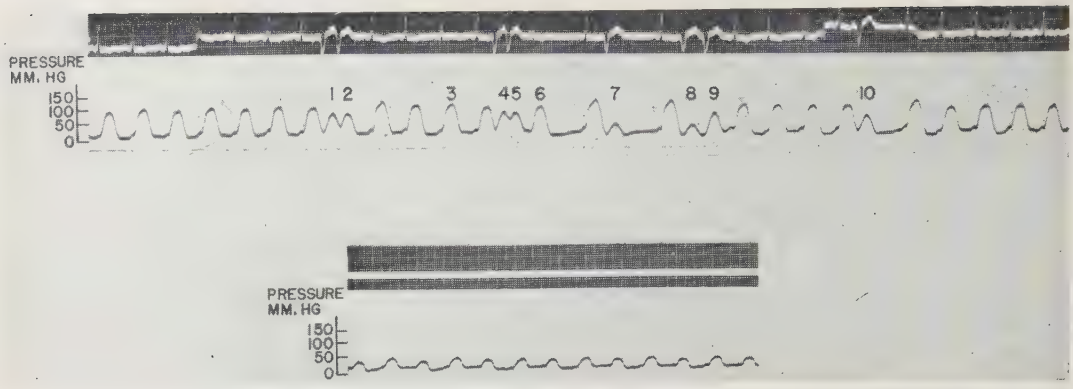

FIG. 1.

Pressure curves of left (above) and right (below) ventricle obtained in a 65-year-old moribund male. Time in each record is in the simultaneously recorded electrocardiographic record (each space between heavy lines equals 0.2 sec.). In the middle section of the upper strip, 1 to 10 indicate the successive premature systoles; 3 is a fusion beat. Discussed in text.

In accordance with Wigger's rule[2] the amplitude of the pressure curve of the post-extrasystolic beat is an inverse function of the duration of the immediately preceding diastole. Thus the magnitude of the pressure pulse occurring after the first pair of premature systoles (beats 1 and 2) is greater than the pressure pulse occurring before the premature systoles. This increase in size of the pressure pulse is more marked after the next two pauses (after beats 6 and 7) and after the last pause (after beat 10). Because there is no pause after the last pair of premature systoles (beats 8 and 9), the size of the post-extrasystolic beat is not significantly larger than the beats that follow. Following the last pause (after beat 10) there is a temporary mechanical alternans in that the second beat after this pause is smaller than the third. The beat registered at the time of the fusion complex (beat 3) is no different than the others. This evidence tends to confirm for man what has been established in the experimental animal that the magnitude of the pressure pulse varies with the duration of the preceding diastole.

The fact that the premature systoles occur at varying time intervals from the preceding beat accounts for the varying size and summation of their pressure curves. The shorter the R-R interval preceding the premature beat the higher up on the pressure curve of the preceding beat does the premature beat begin. The height of the systolic pressure obtained however, is not determined exclusively by the preceding R-R interval. Thus the second and third beats in a run of premature systoles (beats 6 and 9) unexpectedly may have a higher systolic pressure than other immediately preceding premature beats in the same runs (beats 4 and 8). This might be explained by the fact that the first premature systoles are less effective in emptying the ventricular content so that the systolic residue added to the next filling makes the subsequent beat more effective. Furthermore, the duration of the contraction curve of the premature systole is shorter than the normal beat permitting more time for filling before the next premature beat. It may be, however, that this phenomenon is due to the presence of a supernormal phase expressing itself in an increased contractility of the second premature beat.

Another interesting phenomenon is revealed in the pressure curves obtained in the first (beats 1 and 2) and second (beats 4 and 5) runs of the premature systoles. These pressure curves may be said to resemble a summation curve such as is obtained with skeletal muscle. As a matter of fact the pressure during the first two premature beats of the second run (beats 4 and 5) decreases very little; in this instance, therefore, the heart has for practical purposes changed its activity

[2] Wiggers, C. J., *Am. J. Physiol.*, 1925, **72**, 188.

from its normal creation of pressure fluctuation intermittently to maintaining a sustained pressure rise. This suggests the possibility that a rapid run of ventricular premature systoles could leave the heart more or less in sustained systole.

Summary. The recording of direct pressure curves, in which technical errors are reduced to a minimum, from the ventricular cavities of the heart in man emphasizes the great need of interpreting with care pressure curves obtained by other means in this newly expanded field of human physiology.

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17296. Spiral Arterial Structures in the Fetal Placenta.

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Spiraled arterial vessels are known to exist in the uterus[1,2] and ovary.[3,4] In the course of studying the fetal vascular components of the human placenta, the spiral nature of the primary branches of the allanto chorionic vessels has been noted for the first time. These appear to penetrate the substance of the placenta in much the same way as the radiate arteries do the myometrium of the uterus. The fetal spiral arteries arise from the major subchorionic vessels and represent the main vascular channels for the primary villus stem.

One hundred and twenty-five born placentae have been injected with several different masses and the vascular ramifications of the placental fetal vessels are being studied with a variety of technics. Each injection mass has been introduced via a cannulated umbilical artery shortly after the expulsion of the placenta. Fifty of these have been injected either with a 28% or 12% solution of vinyl acetate. The more concentrated solution was injected at 200-250 mm Hg while the more dilute monomer was injected at 120 mm Hg. The placentae were corroded in commercial concentrated hydrochloric acid for 24-48 hours and in turn washed with jets of tap water. The plastic cast of the fetal vessels which was subsequently recovered represented an accurate 3 dimensional model of the vasculature. Serial reconstruction by comparison is a time consuming, laborious task which does not yield as good a result.

The spiral features can be demonstrated more easily in preparations made with the more dilute vinyl acetate injected at physiological pressures. It is our impression that the greater pressure head which is required to completely inject the more concentrated solution straightens out some of the arterial coils. The remainder of the placental preparations, comprising 3 groups of 25 each, were injected at physiological pressures with radio opaque gelatin mass, liquid latex, and India ink, respectively. Each of these masses has advantages as well as distinct limitations. In the overall study, the desirable features of each has been utilized to reconstruct the details of the vascular pattern. The gelatin and India ink injected placentae have been cleared with a modified Spalteholtz technic and then selected areas have been serially sectioned for histologic detail. Liquid latex can be made to set within the vessel wall without distortion of the lumen, diameter, or calibre of the vessel. Such preparations permit a geometric

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1 Hunter, Williams, Anatoma uteri humani gravidi tabulis illustrata, 1774.

2 Daron, G. H., *Am. J. Anat.*, 1936, **58**, 349.

3 Reynolds, S. R. M., *Am. J. Obst. and Gynecol.*, 1947, **53**, 221.

4 Delson, B., Lubin, S., and Reynolds, S. R. M., *Endocrinology*, 1948, **42**, 124.

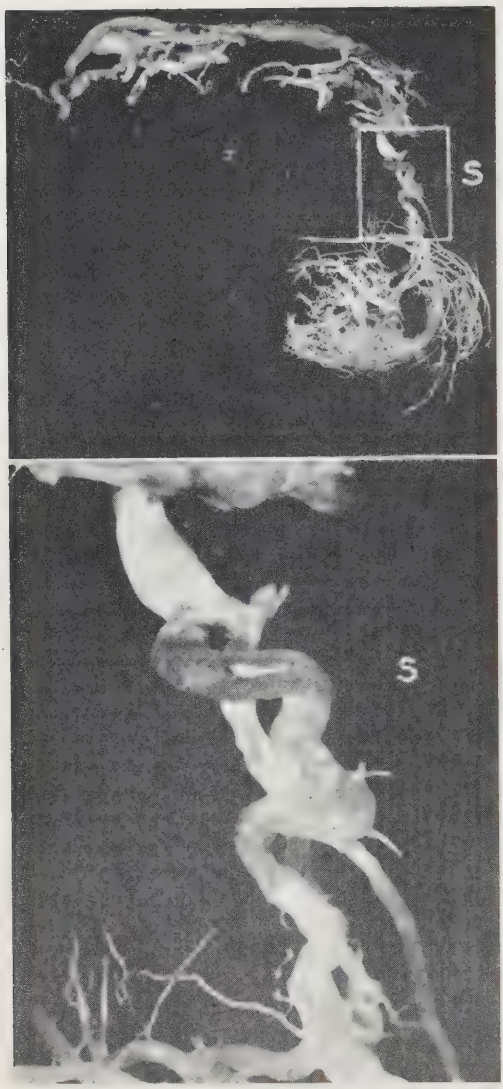

FIG. 1a (top).
Spiraled fetal placental vessels. A corroded plastic preparation revealing arterial coil and partly, paid out arterial coil of primary branch of chorio allantoic vessels. × 3.

FIG. 1b (bottom).
Magnified view of coiling and uncoiling of spiraled fetal vessel shown in area S (Fig. 1a). × 25.

analysis and provide some insight into the hemodynamics of the placental circulation.

All of the preparations reveal aspects of spiraling. Fig. 1 is a corroded plastic cast in which an attempt has been made to isolate by dissection a single allanto-chorionic vessel with its primary spiraled branch and terminal cotyledonary tuft. The spiraled primary villus vessel arises at a right angle to the chorionic plate from the large subchorionic vessels. It forms a helix of slowly diminishing diameter as it passes towards the decidual plate. The number of coils varies considerably averaging between 2 and 5. Some of these appear to be uncoiling and others almost completely paid out. There is a rather marked decrease in the relative diameters between the terminal spiraled segments and the delicate vascular components of the cotyledonary tuft.

Discussion. Physiologic considerations fail to support present concepts of morphologic adaptation of the utero-placental vasculature. Placental transfer is poorly understood, and what information is available fails to support a simple diffusion exchange.[5] A reinvestigation of the essential morphologic features of the fetal vasculature in the placenta has been initiated in order to reevaluate our present concepts. Spiraled arterial and arteriolar vessels in the uterus and ovary are considered to reflect a trophic response to the presence of steroid hormones.[6,7] The mechanism by which this particular morphologic feature of fetal placental arterial spiraling arises is not known. The human placenta is well known as a site of abundant formation of steroid hormones throughout the course of pregnancy.[8] The occurrence of similar spiraled vascular structures, in placental stroma, by analogy, may be attributable to a similar response.

The finding of a helical arrangement on the fetal aspect of the utero placental circulation draws attention to the spiral tuft arrangement of the maternal vessels which tap into the intervillous space. These were first described by Spanner,[9] who contended that this

[5] Barcroft, J., Researches on Prenatal Life, Charles C. Thomas, 1947.

[6] Reynolds, S. R. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 96.

[7] Okkels, H., and Engle, E. T., *Acta path. et microbiol. Scandinav.*, 1938, **15**, 150.

[8] Wislocki, G. B., and Bennett, H. S., *Am. J. Anat.*, 1943, **73**, 335.

[9] Spanner, R., *Z. f. Anatomie*, 1936, **105**, 163.

arrangement provided for a gradient of pressure which allowed for a continuous uninterrupted flow into the intervillous space. The fetal spiral arrangement suggests a similar functional adaptation which can provide for maximal fetal vascular exchange.

Based upon studies of hydrostatic conditions and maternal blood flow in rabbit uteri during pregnancy, Reynolds has elaborated a concept of uterine accommodation. In the last third of pregnancy the period of uterine growth is supplanted by a period of uterine stretching which follows upon an abrupt conversion of the conceptus from spherical to cylindrical form.[10–13] Ramsey has indicated that a transition is to be observed in the maternal vessels of the endometrium of the pregnant rhesus monkey beyond the 52nd gestational day. The coils of the arteries are paid out, corresponding to the period of uterine stretching.[14] The finding of partially uncoiled paid out spirals of placental fetal vessels in mature term placentae is consistent with these observations. The placental vessels must be subjected to the same hydrostatic conditions and mechanical stretching.

Summary. The spiral nature of the primary villus stem vessels has been described for the first time. The underlying mechanism suggests a trophic response to steroid hormones, comparable to the effect produced on vessels of the uterus and ovary. This spiral pattern provides a gradient of pressure which may slow fetal circulation through the placenta and allow for a more thorough exchange.

We are greatly indebted to Dr. S. R. M. Reynolds and Mr. Chester Reather of the Carnegie Institution of Washington, Baltimore, Md., for much valuable assistance. Dr. Reynolds has confirmed some of our observations and offered many helpful suggestions. Mr. Reather has provided us with excellent photographic material.

10 Reynolds, S. R. M., *Anat. Rec.*, 1946, **95**, 283.

11 Reynolds, S. R. M., *Am. J. Physiol.*, 1947, **148**, 77.

12 Reynolds, S. R. M., *Am. J. Obstet. and Gynecol.*, 1947, **53**, 901.

13 Reynolds, S. R. M., Carnegie Inst. Wash. Pub. 583, "*Contributions to Embryology*," 1948, **33**, 1.

14 Ramsey, E. M., Carnegie Inst. Wash. Pub. 583, "*Contributions to Embryology*," 1948, **33**, 113.

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17297. Effects of Intravenous Injection in Dogs of Staphylokinase and Dog Serum Fibrinolysin.*

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Utilizing methods described in previous reports,[1] we have studied the *in vivo* effects of staphylokinase and dog serum lysin (fibrinolysin, plasmin, tryptase) in dogs. Normal dog serum contains the inactive precursor, *prolysin,* of an active fibrinolytic enzyme, *lysin,* and substance(s), *antilysin,* capable of inhibiting this active lysin† Staphylokinase,[2,3] a material obtained from staphylococcal culture filtrates, is capable, *in vitro,* of activating dog prolysin to lysin. It seemed of some interest to determine the effects of this material injected intravenously, as well as those of a potent fibrinolytic enzyme solution, prepared from dog serum by fractionation at 25%

* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

1 Lewis, J. H., and Ferguson, J. H., *Abstr. in Fed. Proc.*, 1949, **8**, 96.

† This terminology is used for brevity.

2 Gerheim, E. B., Ferguson, J. H., Travis, B. L., Johnston, C. L., and Boyles, P. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 246.

3 Lack, C. H., *Nature*, 1948, **161**, 559.

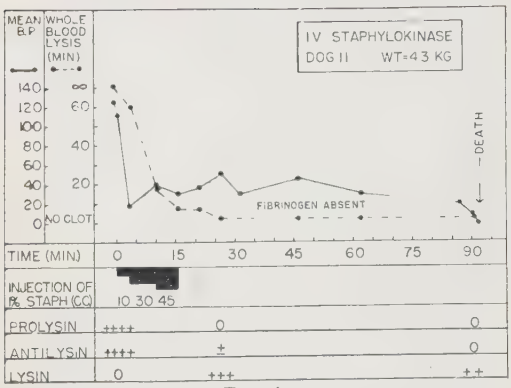

FIG. 1.

saturation with alcohol, treatment with chloroform, and aging for 6 weeks at 0°C. Due to the large amounts of these materials calculated as necessary to reproduce *in vivo* the established *in vitro* effects, we have studied only 2 dogs.

Injection of staphylokinase. 85 cc of a filtered 1% solution of partially purified staphylokinase was injected over a period of 16 minutes into a small fasting mongrel dog previously anesthetized with nembutal.

Fig. 1 summarizes the results observed in this dog. Arterial pressure fell precipitously following the injection of only a few cc. The injection was slowly continued and arterial pressure rose slightly, remaining between 30 and 60 mm Hg until the terminal fall. No whole blood lysis was observed in a pre-injection sample. After 10 cc of staphylokinase had been administered the whole blood lysis time was 60 minutes. As the injection continued, lysis times became shorter until at 10 minutes after the end of the injection lysis could not be measured as this blood did not clot. No significant changes were observed in whole blood coagulation times until this and subsequent specimens which did not clot even on addition of potent thrombin suggesting complete absence of fibrinogen probably caused by *in vivo* fibrinogenolysis.

Three serum samples were studied and results reported in Table I. The control specimen showed no active lysin, normal content of prolysin, as tested by *in vitro* activation with added staphylokinase, and normal antilysin titer. Serum obtained from blood taken 10 minutes after the injection showed marked lysin activity, no demonstrable prolysin (as indicated by the fact that the *in vitro* staphylokinase activation prolonged rather than shortened the lysis time), and markedly diminished antilysin. Immediately before death (90 min.) the serum still showed rather marked lysin content, no prolysin, and no appreciable antilysin. Prolysin times longer than lysin times were due to spontaneous loss of lysin which occurred during the 20 minute incubation at 37°C required for complete activation of prolysin by staphylokinase. In whole blood and serum samples obtained 10 minutes post injection and subsequently, a marked lipemia was noted. Microscopically, the globules were seen to stain readily with Sudan IV.

Injection of dog serum lysin. In vitro studies on the dog serum lysin showed it to be an extremely potent material containing approximately 4000 lysin units per cc. In order to allow sufficient lysin to combine with the calculated amount of circulating antilysin and leave a lysin excess detectable in the serum, we administered 1,000,000 units (250 cc of lysin solution) over a period of 11 minutes. Additional *in vitro* studies showed that this preparation of dog serum lysin contained a small amount of thrombin.

In an effort to minimize possible overloading of the heart due to the large volume of fluid introduced, we removed blood samples totalling 100 cc during the injection period.

Fig. 2 summarizes the observed findings in this 4.2 kg animal. Arterial pressure fell only gradually during and after the injection.

TABLE I.
Serum Lysin, Prolysin and Antilysin after Injection of Staphylokinase.

	Lysin[1]	Prolysin[2]	Antilysin[3]
Control	∞	3½ min.	2460 units
10 min.	8 min.	13½ "	40 "
90 "	13 "	21 "	10 "

[1] Lysis time of standard clot containing 0.5 cc serum.

[2] Lysis time of standard clot containing 0.5 cc serum after activation with staphylokinase.

[3] Calculated from lysis time of standard clot containing 0.5 cc serum previously incubated with 0.5 cc standard lysin.

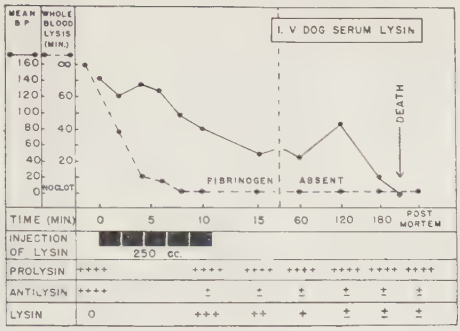

FIG. 2.

Pulse rate was noted to rise markedly during the injection. Whole blood coagulation and lysis times before, during, and following the injection are shown in Table II. The clotting times observed in the infusion period were significantly shortened at first, but after 200 cc of lysin had been introduced no clot was formed even on addition of thrombin, indicating complete fibrinogenolysis. Clot lysis times became progressively shorter until incoagulable blood was obtained.

TABLE II.
Whole Blood Coagulation Times and Lysis Times after Injection of Dog Serum Lysin.

	Clotting times (min.)	Lysis times (min.)
Control	7′	∞
Inj. after 50 cc	1′	39
" " 100 "	1′	8½
" " 150 "	4′*	6
" " 200 "	No clot	—
" " 250 "	" "	—
Min. after inj.		
5′	No clot	—
↓	↓	
180		—

* Never solid.

TABLE III.
Serum Lysin, Prolysin and Antilysin after Administration of Dog Serum Lysin.

	Lysin[1]	Prolysin[1]	Antilysin[1]
Control	∞	3¼ min.	2300 units
After 150 cc	18 min.	3½ "	300 "
" 200 "	17½ "	3½ "	290 "
" 250 "	16 "	3¼ "	230 "
Min. after inj.			
5	24 min.	3½ min.	180 units
15	42 "	3½ "	320 "
30	90 "	3½ "	180 "
60	3-12 hr	3½ "	206 "
120	3-12 "	3½ "	252 "
180	3-12 "	3½ "	320 "
Postmortem	3-12 "	3½ "	246 "

[1] See footnotes Table I.

Table III presents the serum findings in this dog. Control serum showed no active lysin, normal prolysin and normal antilysin. During and following the injection, moderate amounts of lysin appeared and gradually diminished to a trace by one hour. Prolysin was apparently not affected as no significant changes were observed on *in vitro* activation with staphylokinase. Antilysin fell dramatically but, in spite of the presence of demonstrable active lysin, the antilysin titer never reached zero. The experiment was terminated after 3½ hours. Gross postmortem examination was performed. Blood was fluid in the heart and all large vessels examined. The heart was contracted, and, on opening, showed widespread hemorrhagic areas over the endocardium, particularly of the left ventricle. The lungs were congested and showed hemorrhagic areas in both lower lobes. Small ecchymoses were scattered over the serosal surface of the stomach. Liver and spleen appeared grossly normal. No lipemia was noted in this dog.

Discussion. As might be expected from known *in vitro* reactions, both staphylokinase and serum lysin, when injected, resulted in the appearance of active lysin in the withdrawn serum. Evidence of *in vivo* proteolytic activity was seen in the appearance of incoagulable blood in which no fibrinogen could be detected. The mechanisms by which these results were obtained are obviously different. Staphylokinase activated the animal's own prolysin to lysin as indicated by the fact that the prolysin titer decreased as active lysin appeared in the serum. The antilysin titer also fell, indicating that enough active lysin was formed to destroy or combine with the circulating antilysin and still leave an excess of enzyme detectable in the serum. The injection of dog serum lysin, on the other hand, apparently did not affect the circulating prolysin, but the quantity administered was sufficient to neutralize available antilysin leaving excess lysin.

No antilysin titers of zero were obtained. This is probably due to interpretation of the results produced by this method. We are unable to obtain a serum control free from antilysin and, therefore, use simply a borate buffer control. We know that lysin is a proteolytic enzyme not specific for fibrin or fibrinogen as it also attacks casein, gelatin, etc. Other proteins in serum may exert minor nonspecific inhibitory effects by competing with the test substrate, fibrin.

Staphylokinase exerted a marked vasodepressor effect which may be associated with some other toxic material in the culture filtrate. Serum lysin showed lesser vasodepressor effects. Guest *et al.*[4] found marked vasodepressor effects following injection of their fibrinolysin[5] which was prepared, by a different method, from bovine serum. These authors found no changes in blood coagulation and did not report any lytic effects. It should be pointed out, however, that our experiment employed a much larger quantity of enzyme.

Some comment should be made on the marked shortening of coagulation time, from 7 minutes to one minute, immediately following administration of the first 50 cc of dog serum lysin. Numerous experiments, in which repeated arterial sampling from a siliconed cannula has been employed to study blood coagulation times in similarly anesthetized dogs, have frequently shown wide variations between 10 and 3½ minutes, with a tendency to become shorter in spite of repeated saline irrigations of the cannula and discarding of the first few cc of blood at each sampling. For this reason we did not feel that the slight shortenings of clotting-time observed after injection of staphylokinase were significant. On the other hand, the marked drop in clotting-time following injection of serum lysin cannot be dismissed as an experimental error. This is possibly due to the effects of small amounts of thrombin, which were present as a contaminant of this preparation of dog serum lysin.

Conclusions. 1. Staphylokinase, injected intravenously into a dog, caused appearance of active serum lysin, accompanied by a fall in prolysin and antilysin. The blood became incoagulable presumably due to lysis of the circulating fibrinogen. A precipitous fall in blood pressure was also observed.

2. Dog serum lysin, injected intravenously into a dog, also resulted in the appearance of active serum lysin, fall in antilysin, but no appreciable change in prolysin. Incoagulable blood, devoid of fibrinogen, was again obtained.

4 Guest, M. M., Murphy, R. C., Bodnar, S. R., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, **150**, 471.

5 Loomis, E. C., George, J. C., and Ryder A., *Arch. Biochem.*, 1947, **12**, 1.

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17298. Lethal Effect of Triethylene Glycol Vapor on Air-Borne Mumps Virus and Newcastle Disease Virus.*

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Previous laboratory investigations by Robertson and his co-workers,[1,2] and by Henle and Zellat[3] demonstrated that both propylene and triethylene glycol vapor were lethal for air-borne Influenza A virus. Subsequently, Rosebury and his associates[4] showed that triethylene glycol vapor was effective against air-borne meningopneumonitis and psittacosis

* Aided by grants from the U. S. Public Health Service, Carbide and Carbon Chemicals Corporation, and the Research Corporation.

† National Institutes of Health Postdoctorate Research Fellow.

1 Robertson, O. H., Loosli, C. G., Puck, T. T., Bigg, E., and Miller, B. F., *Science*, 1941, **94**, 612.

2 Robertson, O. H., Puck, T. T., Lemon, H. F., Loosli, C. G., *Science*, 1943, **97**, 142.

virus. These observations indicated that the glycol vapors were virucidal as well as bactericidal.[2,3] The experiments to be described demonstrate that triethylene glycol vapor is lethal for two more viral agents, mumps virus and Newcastle disease virus.

Materials and methods. Viruses. A strain of mumps virus‡ was cultivated in the allantoic sac of 9 day old chick embryos which, after inoculation, were incubated at 35°C for 6 days. The infected embryos were then chilled over night at 4°C and the allantoic fluid removed.

The strain of Newcastle disease virus‡ was cultivated in the allantoic sac of 11 day old embryos, which after inoculation, were incubated at 37°C for 52 hours. Infected allantoic fluid was harvested after chilling overnight at 4°C.

Virus Infectivity Titrations. These were performed on the infected allantoic fluids which were to be sprayed into the chamber. Serial tenfold dilutions from 10^{-5} to 10^{-10} were made in sterile broth. A volume of 0.1 cc of the dilution of virus was inoculated through a small drill hole in the egg shell directly over the allantoic sac. Four embryos were used for each virus dilution. Each allantoic fluid was tested for its capacity to agglutinate chicken erythrocytes using the method of Salk.[6] Virus titration end points (E.I. 50)§ were calculated by the 50% end point method of Reed and Muench.[7]

The Glycol Chamber.‖, constructed of wood and glass, (Fig. 1), had a capacity of 5.5 cubic feet. The air was gently circulated by

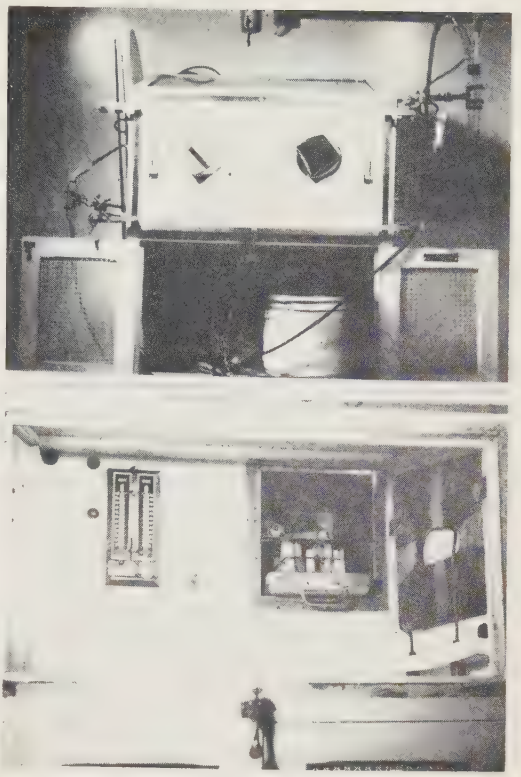

FIG. 1.

a small fan in the left rear compartment. In front of the fan was a Screw Base Resistance Element (10 watts) which served as a source of heat to vaporize glycol impregnated paper. On the left wall of the chamber there were a series of circular coils through which ice water was circulated by means of an electrical water pump. This cooling apparatus was controlled by a thermostat set at the desired temperature.

Viral suspensions were atomized into the chamber through a port on the right side. Air bubbler samples were taken through a port on the left side. A measured volume of air, as indicated by a flowmeter,[8] was withdrawn into a suitable medium for trapping the infective agent. A Welch air pump was used to

[3] Henle, W., and Zellat, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 544.

[4] Rosebury, T., Meiklejohn, G., Kingsland, L. C., and Boldt, M. H., *J. Exp. Med.*, 1947, **85**, 65.

[5] Robertson, O. H., Bigg, E., Puck, T. T., and Miller, B. F., *J. Exp. Med.*, 1942, **75**, 593.

‡ We are indebted to Dr. Frank L. Horsfall, Jr., of the Rockefeller Institute for Medical Research, for the allantoic strain of mumps virus, and to Dr. Alfred S. Evans of Yale University, for the Newcastle disease virus.

[6] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

§ 50% embryo infectivity end point.

[7] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

‖ We are indebted to Dr. Charles C. Chapple of Philadelphia and to Mr. J. W. Spiezelman of Air Purification Service, Inc., for designing the Glycol Chamber.

[8] Lemon, H. M., and Wise, H., *Science*, 1944, **99**, 43.

provide positive pressure for atomization of the suspension and negative pressure for withdrawal of air through a bubbler sampler.[9]

The front partition of the chamber was removable and had two circular perforations 5 inches in diameter. The one on the right was sealed with a cylindrical double air lock which could permit the entry of animals of the size of guinea pigs. The one on the left was sealed by a large rubber gloved sleeve which extended into the chamber, and which could be used for exposing settling plates at periodic intervals.

Triethylene glycol was vaporized into this chamber by placing a strip of impregnated paper on the heating element. This paper¶ contained 0.03 g of triethylene glycol per square inch. The glycol vaporized rapidly and saturated the air of the chamber.

A hygrometer consisting of a dry bulb and wet bulb was located on the back wall of the chamber. The air stream produced by the fan passed over the wet bulb and thus a fairly accurate determination of relative humidity was obtained.

Experimental. Preliminary experiments were conducted to determine if mumps virus could be rendered air-borne, and if so, for how long it could be recovered from the air. Infected allantoic fluid was sprayed into the chamber. Immediately after cessation of spray, serial air bubbler samples were taken for a 15 minute period. An aliquot of each sample was inoculated into the allantoic cavity of 9 day old chick embryos. After 6 days of incubation at 35°C, the fluid was harvested following a preliminary period chilling at 4°C. Each fluid was then tested for its capacity to agglutinate chicken erythrocytes. The results of this procedure indicated that mumps virus could be recovered from the air of the chamber for at least 12 minutes after cessation of spray. This was considered to be an adequate control, and the following experiments with triethylene glycol vapor were then performed.

Effect of Triethylene Glycol Vapor on Air-Borne Mumps Virus. Control Test. 2 cc of allantoic fluid with an E.I. 50 titer of 10^{-8} was delivered by a Graeser atomizer**[10] attached to the inlet port of the chamber. Immediately after cessation of the spray, 2 minute serial air bubbler samples were withdrawn for a 15 minute period. Each bubbler contained 10 cc of 10% horse serum in neopeptone broth. After sampling, each bubbler was treated with penicillin (500 U/cc) and streptomycin (3 mg/cc). An aliquot (0.1 cc) of each sample was inoculated intra-allantoically into 4 chick embryos. After 6 days of incubation at 35°C, the allantoic fluids were harvested. Hemagglutination titrations were then performed on the individual allantoic fluids.

Glycol Test. After completion of the control test the chamber was saturated with triethylene glycol vapor. The identical procedure was then repeated.

Environmental Conditions. The temperature was 83°F (28°C) and the relative humidity was 36% in both the control and glycol tests.

Results. Mumps virus was obtained from the air of the control chamber for at least 13 minutes after cessation of spray. When the air of the chamber was saturated with triethylene glycol vapor, no mumps virus could be recovered (Table I). In 2 of 4 experiments no virus could be detected even immediately after cessation of spray. In the other 2 confirmatory experiments, virus was recovered only in the first sample taken immediately after completion of spray, but at no time thereafter.

In order to determine whether negative hemagglutination represented absence of virus or low titer virus, the following procedure was carried out. The negative allantoic fluids of each sample were pooled and each pool was inoculated intra-allantoically into 4 chick embryos. After incubation and harvesting, the individual allantoic fluids were tested for their capacity to agglutinate chicken erythro-

[9] Lemon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 298.

¶ Prepared by Air Purification Service, Inc., Newark, N. J.

** Delivers a particle size of 1.7 μ mean mass diameter.

[10] Graeser, J. B., and Rowe, A. H., *Am. J. Dis. Child.*, 1936, **52**, 92.

TABLE I.
Effect of Triethylene Glycol Vapor on Air-Borne Mumps Virus.

Time interval min. after spray	Control test Hemagglutination titer* Individual allantoic fluids				Mean	Glycol test Hemagglutination titer* Individual allantoic fluids				Mean
0-2	5120	5120	5120	160	2560	0	0	0	0	0
3-5	160	160	160	640	240	0	0	0	0	0
6-8	160	0	640	80	80	0	0	0	0	0
10-12	0	320	0	1280	60	0	0	0	0	0
13-15	0	1280	0	80	40	0	0	0	0	0

* Expressed as reciprocal.

TABLE II.
Effect of Triethylene Glycol Vapor on Air-Borne Newcastle Disease Virus.

Time interval min. after spray	Control test Hemagglutination titer* Individual allantoic fluids				Mean	Glycol test Hemagglutination titer* Individual allantoic fluids				Mean
0-½	5120	5120	1280	5120	3840	0	0	0	0	0
1-1½	5120	5120	5120	—	5120	0	0	0	0	0
2-2½	5120	5120	1280	5120	3840	0	0	0	0	0
5-5½	5120	5120	5120	5120	5120	0	0	0	0	0
10-10½	640	0	5120	—	265	—	—	—	—	—
15-15½	0	0	0	—	0	—	—	—	—	—

* Expressed as reciprocal.
— Not done.

cytes. This hemagglutination test was completely negative and was interpreted as indicating absence of virus rather than low titer virus.

In the first experiment with mumps virus, the agent was detected in all samples during the 15 minutes control period. In the presence of a saturated atmosphere of triethylene glycol vapor, no virus could be recovered. The chamber was then cleaned and aired for a 2 week period. When the same experiment was repeated, mumps virus could only be recovered from the first (0-2 min.) sample in the control test. It seemed possible that the minute amount of residual glycol, which had condensed on the wall of the chamber, was sufficient to interfere with the control test. There was no visible vapor and one can only guess that the percentage saturation of triethylene glycol vapor in the chamber air was very low. After heating the interior of the chamber with a 200 watt bulb to vaporize all of the residual glycol, the original experiment was easily repeated and confirmed. These observations suggest that triethylene glycol vapor is lethal for mumps virus in concentrations below saturation.

Effect of Triethylene Glycol Vapor on Air-Borne Newcastle Disease Virus. The virus infectivity titration of the allantoic fluid was 10^{-8} E.I. 50. This material was used for a control test and a glycol test. The procedure was the same as that used in the mumps experiments with the following modifications: 1) 1½ cc of Newcastle disease virus was atomized into the chamber. 2) ½ minute air bubbler samples were taken. 3) Each bubbler contained 8 cc of 1% Casamino acids. 4) The temperature was 74°F (23°C) and the relative humidity was 50% during both the control and glycol tests.

Results. As indicated in Table II, Newcastle disease virus could be obtained from the air of the control chamber for 10 minutes after cessation of spray. No virus could be recovered when the chamber was saturated with triethylene glycol vapor.

Discussion. In this study, the hemagglutination test has been used as a measure of virus concentration. Beveridge and Lind[12] found that there was a direct relation between

[12] Beveridge, W. I. B., and Lind, P. E., *Australian J. Exp. Biol. and Med. Sc.*, 1946, **24**, 127.

the hemagglutination and complement fixation titers of mumps infected allantoic and amniotic fluids and concluded that the hemagglutination titer was a function of virus concentration. Ginsberg, Goebel and Horsfall[11] showed that the rate of increase in concentration of virus, as determined by infectivity titrations, paralleled the rise of hemagglutination titers. The latter workers also found that an E.I. 50 titer of the order of $10^{-4.30}$ or higher was necessary before hemagglutination with mumps virus was demonstrable.

In order to rule out the possibility of a virus concentration titer too low to be detected by hemagglutination, the allantoic fluids of the glycolized air samples were reinoculated into chick embryos. Following this second passage, no rise in titer was observed, and it was therefore concluded that no mumps virus was present.

Summary. 1. Under the conditions of these experiments, triethylene glycol vapor in saturated concentrations was rapidly lethal for air-borne mumps virus and Newcastle disease virus.

2. Triethylene glycol vapor was also lethal for air-borne mumps virus in concentrations below saturation.

We should like to express our indebtedness to Dr. Robert Ward for his helpful advice throughout this study.

11 Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **87**, 385.

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17299. Effect of Ammonium Chloride on Serum Sodium/Chloride Ratio in Foreign Serum Arteritis in Rabbits.

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An arteritis has been induced in rats by the administration of large amounts of desoxycorticosterone acetate (D.C.A.) by Selye,[1] and in rats so treated the development of these lesions was inhibited when NH_4Cl was added to their diet.[1] D.C.A. overdosage in rats also causes a rise in the serum sodium and the serum Na/Cl ratio[1,2] and a reduction of this ratio toward the norm following NH_4Cl therapy has been held to be significant in inhibiting the development of D.C.A. induced arteritis and other lesions in the rat.[1] It was thought that if this elevation of the serum Na/Cl ratio was an essential step in the pathogenesis of the D.C.A. arteritis, then if there is a common pathogenesis in the development of the D.C.A. arteritis and the well known foreign protein arteritis,[3] there should also be an elevation of the serum sodium and Na/Cl ratio in animals given foreign protein which develop arteritis. It also seemed a logical possibility that ammonium chloride might inhibit the development of foreign protein arteritis if there is a common pathogenesis in the development of these two types of arteritis. The following experiments were designed to determine whether these logical possibilities concerning the pathogenesis of these two types of arteritis are correct.

Materials and methods. Group 1. Eighteen albino rabbits of 2-3 kg weight were given a diet of Purina Rabbit Chow and water to drink *ad libitum*. The rabbits were given an intravenous injection of sterile whole horse serum in a dose of 10 cc/kg of body weight. The serum injection was repeated after 17 days. Seven days later all animals were killed by air embolism and sections were prepared for histological study from all tissues as in our previous studies.[3]

Group 2. Seventeen albino rabbits of 2-3 kg weight derived from the same sources and

1 Selye, H., Hall, O., and Rowley, E. M., *Lancet*, 1945, **248**, 301.

2 Friedman, S. M., Polley, J. R., and Friedman, C. L., *J. Exp. Med.*, 1948, **87**, 329.

3 More, R. H., and McLean, C. R., *Am. J. Path.*, 1949, **25**, 413.

TABLE I.
Serum Sodium/Chloride Ratios Estimated in Milliequivalents.

		A	B	C	D
Group I	Mean	1.50 ± .03	1.49 ± .02	1.41 ± .03	1.47 ± .01
Received serum only	S. Dev.	.10	.06	.08	.04
Group II	Mean	1.51 ± .03	1.39 ± .03	1.32 ± .01	1.31 ± .04
Received serum and NH_4Cl	S. Dev.	.08	.09	.05	.14

A. Before any treatment was given.
B. Immediately before the second serum injection.
C. Two days after the second serum injection.
D. Immediately before termination of the experiment.

living in the animal quarters at the same time were treated in exactly similar manner except that they were given a 0.9% ammonium chloride solution instead of water to drink. The salt solution was supplied in quantities of 300 cc daily; the residue, if any, from each 24 hour period was measured. This salt solution was well tolerated, although the majority of animals gave evidence of thirst and drank most of their daily quota within a few hours of being fed. Ammonium chloride was given for one week before the first injection of horse serum and continued until the end of the experiment.

Serum sodium and chloride levels in 10 animals from each group were determined on 4 occasions: A—before any treatment was given; B—immediately before the second serum injection; C—two days after the second serum injection; D—immediately before termination of the experiment. Sodium determinations were done according to the method of Barber and Koltoff[4] as modified by Butler and Tuthill,[5] and the chloride determinations were done by the method of Wilson and Ball.[6]

Results. The means, standard deviation, and standard error of the means of the serum Na/Cl ratio expressed as milliequivalents, are shown in Table I. The serum sodium was not appreciably affected either by serum treatment or the addition of ammonium chloride to the diet or both. There was no elevation of the Na/Cl ratios of the Group I animals receiving serum alone as occurs in rats given large amounts of D.C.A. The individual animals of this group which developed lesions and on which serum sodium and chloride determinations were made showed no elevation of the serum Na/Cl ratio. In the animals of Group 2 receiving both serum injections and ammonium chloride in their drinking water, there was a progressive elevation of the mean serum chloride, and this was reflected in a depression of the Na/Cl ratio. This was most marked following the second injection of serum, when it reached levels that were significantly lower than normal values for this group of animals before treatment or values in the Group I receiving serum alone determined at corresponding times. Five of the sodium and chloride determinations made on animals from Group 2 receiving both foreign protein and ammonium chloride were made on animals that developed arterial lesions. In two of these the Na/Cl ratio was depressed significantly below normal values.

Focal inflammatory lesions were found in the coronary and visceral arteries of both groups. The arterial changes were qualitatively similar in both groups of animals. These were comparable to the lesions produced by injections of horse serum that we have described in detail elsewhere,[3] and illustrated in Fig. 1. However, there was some difference in the incidence of the arterial lesions between the animals of Group 1 which received horse serum only and those of Group 2 which received both horse serum and ammonium chloride as shown in Table II. Arterial lesions were seen in 10 animals of Group 1 and in 6 of the ammonium chloride treated series. The total number of organs exhibiting arterial lesions in the animals of Group 1 was 23,

[4] Barber, H. H., and Kolthoff, I. M., *J. A. Chem. Soc.*, 1928, **50**, 1625.

[5] Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, **93**, 171.

[6] Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, 1928, **79**, 221.

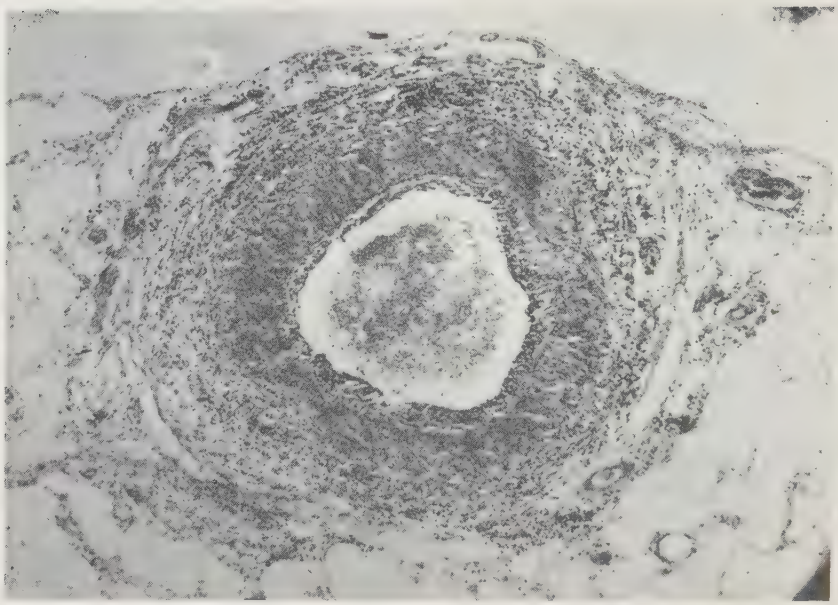

FIG. 1.
Typical arteritis following two large intravenous injections of horse serum, presenting fibrinoid necrosis of the media with cellular exudation into the intima, media and adventitia consisting, essentially, of large mononuclear cells.
Hematin phloxine and saffron × 90.

whereas that in the ammonium chloride treated group was only 11.

Five of the animals of Group 1 and 6 of those of Group 2 showed focal inflammatory lesions of the heart valves. Morphologically, those of the NH_4Cl treated group were indistinguishable from those of Group 1.

Discussion. Selye[1] has postulated that one important factor in the pathogenesis of D.C.A. induced arteritis is the elevation of serum sodium with consequent elevation of the serum Na/Cl ratio. In order to establish the possible relationship between the pathogenesis of D.C.A. induced arteritis and foreign serum protein arteritis, it seemed to us important to establish whether this same relationship of elevated serum Na/Cl ratios and the development of arteritis held for the production of foreign serum protein arteritis. In Group 1 receiving serum only, our results show that arteritis occurred without elevation of the serum sodium or serum Na/Cl ratio. It is clear then that if the elevation of the serum Na/Cl ratio is an essential factor in the pathogenesis of D.C.A. induced arteritis, then the pathogenesis of foreign protein arteritis must be different. Further evidence that an elevation of the Na/Cl ratio is not important in the development of the foreign protein arteritis is indicated by the fact that in the animals receiving both horse serum and ammonium chloride two animals, at least, that developed arteritis, showed a significant reduction in the sodium chloride ratio below the mean normal value and significantly below the control pre-treatment values in these two rabbits. However, in view of the observations of Darrow and Miller[7] that the mineral content may vary independently in the serum and tissues, the possibility still exists that in the present experiment there may have been alterations in the sodium content of the tissues undetected by analysis of serum sodium.

The interpretation of the possible inhibitory effect of ammonium chloride on the development of the arteritis is not so clear. There was a decrease in the total number of affected animals and in the total number of organs with lesions in Group 2, but this decrease was not statistically significant when

[7] Darrow, D. C., and Miller, H. C., *J. Clin. Invest.*, 1942, **21**, 601.

TABLE II.
Comparison of Incidence of Arteritis Between Group I and II.

	Incidence in organs		Incidence in animals	
Arteries	Group I Serum only 18 rabbits	Group II Serum and NH_4Cl 17 rabbits	Group I Serum only 18 rabbits	Group II Serum and NH_4Cl 17 rabbits
Coronary	10	5		
Aorta	6	2		
Mesenteric	2	2		
Gastric	1	1		
Pancreatic	4	1		
Total	23	11	10	6

compared with the incidence of lesions in the animals of Group 1 receiving horse serum alone. Whether the trend might become significant by a more effective use of ammonium chloride is not established from the results, but it seems possible for the following reason. The depression of the Na/Cl ratio did not reach significant levels until after the 24th day of the treatment and this fact may indicate that any effect of ammonium chloride on the animals as a whole was not reached until this time. It therefore seems possible that if ammonium chloride (by whatever mechanism) is responsible for the lowered incidence of lesions seen in Group 2, then more prolonged treatment with ammonium chloride before the animals receive foreign protein injections might be more effective in inhibiting the development of these lesions, and the trend toward inhibition of arteritis in the rabbits receiving both horse serum and ammonium chloride might reach significant levels. If this latter possibility were the case it might indicate a common pathogenesis for the development of the D.C.A. induced arteritis on the one hand and foreign protein induced arteritis on the other, but from what has already been said, the alteration of the Na/Cl ratio could not be an essential step in such a common pathogenesis.

Summary. The possibility was considered that experimentally induced D.C.A. arteritis and foreign protein arteritis might have a common pathogenesis mediated by way of an elevation of the serum Na/Cl ratio. To test this hypothesis one group of animals was given horse serum alone, and another both horse serum and ammonium chloride. There was no elevation of the serum sodium or Na/Cl ratio in those animals receiving horse serum alone and some of the animals receiving both horse serum and ammonium chloride showed a significant depression of the Na/Cl ratio, even though they developed an arteritis.

These results clearly indicate that the development of foreign protein arteritis is not mediated through alterations of the serum sodium chloride ratio and therefore if such an alteration is essential to the development of D.C.A. induced arteritis as suggested by Selye,[1] then the pathogenesis of these two varieties of arteritis must be different. There was a decreased incidence of arteritis in the group of animals receiving horse serum and ammonium chloride, and it is suggested that this trend might become significant by a more effective use of ammonium chloride.

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17300. Protective Effect of Vitamin B_{12} Upon Hepatic Injury Produced by Carbon Tetrachloride.*

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The relation between vitamin B_{12} and ribonucleic acid compounds is, at present, being investigated.[1-4] Vitamin B_{12} has been reported to prevent the disappearance of liver basophilia which occurs when soybean oil meal is the sole source of protein in the diet of rats.[5] This basophilic hue of the cytoplasm of liver cells is due to the presence of ribonucleic acid compounds.[6] In addition, it has been shown that ribonucleic acid decreases or disappears in the initial stages of carbon tetrachloride intoxication,[7,8] as well as in other types of hepatic injury.[9,10] These findings suggested an investigation of the effect of vitamin B_{12} upon acute hepatic injury due to CCl_4 intoxication in rats as an easily reproducible example of hepatic injury.

Methods. A total of 69 female rats, 2 to 3 months old, on a well balanced diet with a relatively high protein content received intraperitoneally 1 cc of mineral oil containing 0.033 cc of CCl_4 per 100 g of body weight. Of these, 38 received 15 μg of vitamin B_{12}‡ per 100 g of body weight, divided into 4 doses, injected subcutaneously 72, 48, and 24 hours prior to, and simultaneously with, the injection of the CCl_4. As controls 7 normal rats of the same strain and age were used for chemical and histological examination of the liver and 25 for the bromsulphalein (BSP) retention test.

To evaluate the degree of liver damage 48 hours after the administration of the CCl_4 (the time of the most marked deposition of fat[11,12]) the following tests were done:

1. Immediately before sacrificing the animals, the serum BSP concentration was determined[13] in 1.5 cc of blood obtained by heart puncture 30 minutes after intraperitoneal administration of 5 mg of dye per 100 g body weight. The results were expressed in mg of BSP per 100 cc of serum.

2. The total liver lipids were determined after extraction with absolute alcohol and reextraction of the alcoholic extract with ether according to Bloor.[14] The results were recorded both in grams per 100 g of liver and milligrams per 100 g of body weight.

3. The amount of fat was graded from 0 to 3+ in Sudan III stained slides of formalin-fixed material. As the fat deposition starts around the central vein, the amount is reflec-

* Supported by grants from the Dr. Jerome D. Solomon Memorial Research Foundation and the Committee on Scientific Research of the American Medical Association.

† Solomon Foundation Fellow.

‡ Vitamin B_{12} concentrate (Rubramin) in ampuls containing the equivalent of 15 μg of vitamin B_{12} was generously supplied by E. R. Squibb & Sons, New York.

1 Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

2 Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

3 Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, 1948, **176**, 1465.

4 Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, 1949, **177**, 995.

5 Stern, J. R., Taylor, M. W., and Russell, W. C., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 551.

6 Dempsey, E. W., and Wislocki, G. B., *Physiol. Rev.*, 1948, **26**, 1.

7 Rosin, A., and Doljanski, L., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 62.

8 Szanto, P. B., and Popper, H., *Bull. Int. Assn. Med. Museums*, 1948, **28**, 119.

9 Opie, E. L., *J. Exp. Med.*, 1946, **84**, 91.

10 Szanto, P. B., and Popper, H., *Proc. Inst. Med. Chicago*, 1948, **17**, 169.

11 Cameron, G. R., and Karunaratne, W. A. E., *J. Path.*, 1936, **42**, 1.

12 Koch-Weser, D., *Sao Paulo Medico*, 1946, *Fevereiro*, 167.

13 Casals, J., and Olitzky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 383.

14 Bloor, W. R., Biochemistry of Fatty Acids, Reinhold Publishing Corp., New York, 1943.

TABLE I.

Bromsulphalein Retention, Total Liver Lipids, Amount of Fat and Ribonucleic Acid Depletion with Standard Deviation in Normal Rats and Rats Intoxicated with CCl_4 with and without Previous Administration of Vitamin B_{12}.

No. of rats	Administration of Vit. B_{12}, μg/100 g	Administration of CCl_4, cc/100 g	BSP retention in mg % Mean	BSP retention in mg % S.D.	Total liver lipids g % liver Mean	g % liver S.D.	mg % body wt Mean	mg % body wt S.D.	Histologic fat grade Mean	Histologic fat grade S.D.	Ribonucleic acid depletion grade Mean	Ribonucleic acid depletion grade S.D.
25			0.29	0.26								
7					4.8	1.4	166	24.2	0.15	0.28	0.65	0.75
31		0.033	2.12	0.97	8.8	1.4	366	61.6	2.00	0.70	3.60	0.87
38	15	0.033	0.90	0.60	6.2	1.4	236	41.5	0.83	0.60	2.50	0.83

ted in the distribution. Therefore the criteria used can simply be described as follows: 0 - absence of fat; 1+ – fat deposition in the central zone; 2+ – fat deposition in the central and intermediary zone; 3+ – diffuse fat deposition.

4. The ribonucleic acid depletion of the cytoplasm was graded from 0 to 5+ in methyl green-pyronine stained slides of Carnoy-fixed material. The specificity of the stain for ribonucleic acids was confirmed by digestion with ribonuclease.[15] Again the distribution was graded as a measure of the intensity of the depletion as follows: 0 — no depletion; 1 + – partial depletion of the central zone; 2 + – marked depletion of the central zone; 3 + – depletion of the central and intermediary zone; 4 + – depletion of the central and intermediary zone and partial depletion of the peripheral zone; 5 + – diffuse depletion.

In addition, hematoxylin-eosin stained sections were studied for confirmation.

Results. The rats intoxicated with CCl_4 showed the characteristic histologic alterations.[11] In comparison to the controls, there was increased deposition of lipids (demonstrated histologically and chemically), increased BSP retention and ribonucleic acid depletion. Intoxicated rats which previously had received vitamin B_{12} showed significantly less histologic alterations than the non-treated, CCl_4 intoxicated rats. In the treated group, the increase in BSP retention and in fat deposition was only one-third and the ribonucleic acid depletion about one half that observed in the non-treated group. In general, the 4 criteria studied quantitatively varied in a parallel fashion in individual animals.

Discussion. The previous administration of vitamin B_{12} protects rats from CCl_4 intoxication to a considerable degree. This is evidenced by 4 criteria selected for quantitative studies, 2 of them histologic and 2 of them biochemical. On the average, the vitamin B_{12} treated animals showed approximately one-third of the changes observed in non-protected rats. Although the criteria selected concerned different hepatic functions, there was marked parallelism in the degree of alterations in the individual animals, the intoxicated as well as the protected. The dose of vitamin B_{12} used was excessively large when compared to the effective dose in anti-anemic therapy. However, the smallest effective dose in hepatic injury is yet to be determined. The dose in absolute weight is much smaller than that of any other substance used in lipotropic or protective therapy of the liver.[16]

The mechanism of action of vitamin B_{12} in ameliorating the effect of CCl_4 intoxication upon the liver is not yet fully elucidated. Since the animals were on a balanced diet, a primary lipotropic effect, comparable to that of choline and methionine, is doubtful; the latter occurs, if at all, only in animals on low protein diet.[17] If, as originally assumed, the protective effect through the ribonucleic acid metabolism could be further confirmed, vitamin B_{12} therapy would be promising in other types of hepatic injury. It is possible

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17 Drill, V. A., and Loomis, T. A., *J. Pharm. Exp. Ther.*, 1947, **90**, 138.

that the influence upon nucleic acids is the basic function of this vitamin. This may be the mechanism of its action as the maturation factor of erythrocytes.

Summary. Administration of 15 μg per 100 g body weight of vitamin B_{12} to rats preceding acute CCl_4 intoxication inhibits the development of histologic changes, especially fatty metamorphosis and depletion of ribonucleic acid. In addition, there is less deposition of lipids determined biochemically and less BSP retention than in the intoxicated controls. These results are tentatively related to an effect of vitamin B_{12} on cytoplasmatic ribonucleic acid, which has been shown to disappear early in hepatic injury.

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17301. Persistence of Desoxycorticosterone-Induced Hypertension in the Nephrectomized Rat.

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The administration of desoxycorticosterone acetate (DCA)* provokes hypertension characterized by persistent elevation of systolic and diastolic blood pressures. This has been observed in rats[1–4] and in dogs.[5] Elevation of blood pressure to hypertensive levels in patients with Addison's disease,[6,7] and also aggravation of essential hypertension[8,9] have been observed following DCA treatment.

Animals treated with this hormone develop nephrosclerosis[1,10] and periarteritis nodosa,[11] vascular lesions which are frequently observed in hypertension induced experimentally by various surgical interventions on the kidney.[12] This raises a question as to the mechanism whereby DCA exerts its effect. The most commonly encountered views are either that DCA causes the development of hypertension which then results in widespread vascular damage, or that the vascular tissue is affected first with hypertension developing as a sequel to interference with intrarenal haemodynamics.[5,13,14]

Whether or not the hypertensive effect of DCA is mediated via the kidneys is a question of fundamental importance in the interpretation of experimental results involving this hormone and is also intimately concerned with the nature of hypertension accompanying adrenal cortical hyperfunction.

Hypertension resulting from the application of a constricting ligature to one renal artery subsides completely and promptly to normotensive levels following total nephrec-

* The desoxycorticosterone acetate used in this experiment was generously provided by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J.

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6 McCullagh, E. P., and Ryan, E. J., *J.A.M.A.*, 1940, **114**, 2530.

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10 Selye, H., and Hall, C. E., *Am. Heart J.*, 1944, **27**, 338.

11 Selye, H., and Pentz, I., *Canad. Med. Assn. J.*, 1943, **49**, 264.

12 Smith, C. C., and Zeek, P., *Am. J. Path.*, 1947, **23**, 147.

13 Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

14 Soffer, L. J., Diseases of the Adrenals, 2nd edition, Lea and Febiger, 1948, 229.

TABLE I.
Effect of Nephrectomy on the Blood Pressure of Hypertensive and Normotensive Rats.

		Blood pressure in mm Hg								
		Before uninephrectomy		Total nephrectomy						
Group	Rat No.	Initial	Final	Before	5 hr	21 hr	30 hr	45 hr	55 hr	72 hr
Control	1	98	112	106	129	119	139	—	—	—
	2	112	125	118	129	122	128	—	—	—
	3	100	123	116	138	124	118	123	148	—
	4	114	115	135	128	149	—	—	—	—
	5	112	139	127	131	150	127	—	—	—
	6	105	116	105	117	124	149	126	—	—
DCA	7	94	120	166	157	158	170	154	187	—
	8	86	115	178	205	183	200	201	191	—
	9	108	107	159	173	166	158	160	187	205
	10	117	124	157	166	156	182	193	187	—
	11	96	114	152	157	164	156	166	—	—
	12	95	106	143	168	149	158	186	151	—
	13	101	117	162	171	191	156	193	193	206

Blood pressures of controls and DCA treated rats at selected intervals during the course of the experiment. Controls animals Nos. 4 and 5 proved to have a mild interstitial nephritis.

tomy.[15] This led the authors to conclude that hypertension of renal origin cannot be maintained in the completely nephrectomized animal. If then, hypertension resulting from DCA treatment is in reality a renal hypertension provoked by a vasculo-toxic effect of DCA on the renal vessels, an immediate return of the blood pressure from hypertensive to normal levels would be expected to occur in the totally nephrectomized DCA treated animal. This hypothesis was tested in the following manner.

Materials and methods. Fourteen young male albino rats weighing between 88-138 g (avg 110 g) constituted the experimental series. For a period of 2 weeks prior to the initiation of DCA treatment several determinations of blood pressure were made in order to accustom the animals to the apparatus and to determine the basal pressure of the rats. During this period they received Purina dog chow and tap water *ad libitum*. On the 15th day all animals were subjected to left nephrectomy under ether anesthesia and divided into two groups. The 8 animals of Group I received subcutaneously two compressed pellets of DCA weighing 50 mg each, while the 6 rats of Group II served as controls. At this time the tap water was replaced by 0.85% NaCl solution which served as drinking fluid for both groups until the second nephrectomy was performed. The food was not altered. The sensitizing influence which uninephrectomy and sodium chloride exert on the hypertensive effect of DCA has been described previously.[11]

Throughout the course of the experiment blood pressure measurements were taken on the animals of both groups several times weekly until total nephrectomy, thereafter twice daily, using the method of Williams, Harrison, and Grollman[16] as modified by Sobin.[17] The measurements were made on unanesthetized animals after a preliminary warming for an adequate period in a specially constructed heating chamber, after which the tail was kept warm by the circulation of heated water through a jacket surrounding the membrane of the plethysmograph. At each determination the blood pressure was measured until two successive readings agreed to within 5 mm Hg then the 4 subsequent readings were taken and averaged. This method has proven to be simple and reliable under the conditions described.

15 Rodbard, S., and Katz, L. N., *Am. J. M. Sc.*, 1939, **198**, 602.

16 Williams, J. R., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

17 Sobin, S. S., *Am. J. Physiol.*, 1947, **146**, 179.

Twenty-eight days after the initiation of DCA treatment the animals of Group I had developed definite hypertension. At this time the remaining kidney was excised from animals of both groups. Inasmuch as both food and water have been shown to exert a detrimental effect on survival after total nephrectomy[18] both were withheld from the animals for the remainder of the experiment. Blood pressure recordings were made on each animal in the early morning and late afternoon until death supervened. The kidney which was removed at the second operation was fixed in Bouin's solution for histologic examination. One of the rats in Group I died before the second operation and was thereby eliminated from the experiment.

Results. The basal blood pressure values prior to uninephrectomy and the institution of DCA treatment were essentially the same in the animals of both groups. Hypertension was observed in some animals of Group I as early as the 14th day after implantation of DCA and was manifest in all of the animals of the group by the 17th day. In accordance with previous experience the animals of Group I consumed a considerably greater quantity of fluid than the controls and developed a marked diuresis. During the period before total nephrectomy the blood pressure of the control animals (see Table I) exceeded 129 mm Hg on only one occasion whereas the animals of Group I on the contrary displayed a constantly rising blood pressure. At the time of total nephrectomy the average blood pressure of the control animals was 118 mm Hg (range 105-127) while that of the treated animals was 160 mm Hg (range 143-178).

Subsequent to total nephrectomy the blood pressure of the animals in Group II remained essentially unaltered at the preoperative normotensive level (Table I and Fig. 1). The only animal in this group which survived for 55 hours had a blood pressure of 148 mm Hg. This single value is probably without significance. Single hypertensive readings were occasionally encountered in other control animals, but no definite hypertensive trend was apparent, a hypertensive reading often being

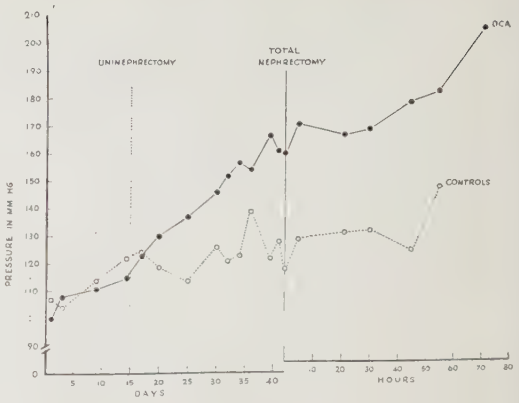

Fig. 1.
Blood pressure of DCA treated and control rats. A progressive rise in blood pressure to hypertensive levels followed the implantation of DCA on the 15th day. This was not abolished by total nephrectomy.

followed by one within the normal range. The reason for this is not clear. Further work is in progress to elucidate the reason for this behavior. Postoperatively the blood pressure of the animals of Group I rose from an average of 160 mm Hg to one of 178 mm Hg at 55 hours at which time the group was still intact, and to 206 mm Hg at 72 hours when 2 animals survived.

The results, summarized in Fig. 1, indicate that rather than declining after total nephrectomy the blood pressure of the animals of the DCA treated group continued to rise, while that of the control animals remained unaltered.

In accordance with previous findings[19] DCA prolonged the survival time of the completely nephrectomized animal (Table I). No attempt was made to accurately determine the time of death in these animals.

Histologic examination of the kidney which had remained *in situ* throughout the period of DCA treatment failed to reveal the presence of vascular lesions. In some of them a few convoluted tubules were dilated and an occasional cast was observed. Two of the control animals (Table I) exhibited interstitial nephritis of a mild degree.

Discussion. Hypertension is one of the most commonly encountered symptoms in

[18] Bergman, H. C., and Drury, D. R., *J. Clin. Invest.*, 1939, **18**, 777.

[19] Selye, H., *Canad. Med. Assn. J.*, 1940, **43**, 333.

clinical cases of adrenal cortical hyperfunction, especially of the Cushing's disease type. In such cases, just as in chronic essential hypertension, renal arteriosclerosis (nephrosclerosis) is usually an associated manifestation. In renal hypertension produced by the application of a partially constricting ligature to one renal artery, again hypertension and nephrosclerosis are present.[20] Therefore there is a school of thought which holds the hypertension of adrenal cortical hyperfunction and of essential hypertension to be due to renal mechanisms. The belief that essential hypertension is a renal disease has led to numerous attempts to relieve or cure the disease by uninephrectomy. The successes following such operations[21-23] have been paralleled by the failures,[24-26] the latter occurring even when tests revealed the remaining kidney to be normal. Thus the role of the kidney in the genesis and maintenance of essential hypertension is uncertain. However, since in animals with experimental renal hypertension removal of the ligated kidney will result, at least in the acute phase, in abolition of the hypertension, it is reasonable to assume that if DCA induces hypertension by a renal mechanism this should be "cured" by nephrectomy.

The role of the adrenal cortex, from which desoxycorticosterone may be extracted,[27] in essential hypertension is similarly unsettled. A high degree of correlation between the occurrence of adrenal cortical adenomas and essential hypertension has been reported by some observers, but others have not noted the association.[13] Similar disagreement exists as to the status of urinary cortin in essential hypertension. The methods at present in use permit separation of the true corticoids, which may be mineral-active or carbohydrate-active, from testoid cortical hormones, but they do not sharply separate the individual hormones from one another. It may be that the cortical hormone or hormones involved in essential hypertension escape detection by current methods.

The present data indicate that the kidneys are not necessary participants in the maintenance of DCA hypertension.

This experiment does not explain the mechanism whereby DCA exerts a hypertensive effect, but it does indicate that the hormone does not induce hypertension by evoking the renin-angiotonin system. On similar grounds the hypertension of early adrenal cortical hyperactivity is probably of non-renal origin. It is possible, however, that when the nephrosclerosis develops, whether in the DCA treated animal or in the clinical conditions of essential or adrenal cortical hypertension, a renal hypertension may become superimposed upon a non-renal form.

Absence of vascular lesions in the acutely hypertensive DCA treated rat and their invariable presence in the chronically treated rat suggests that hypertension precedes nephrosclerosis in development. Therefore either hypertension causes the renal vascular lesions or the two are independent manifestations of DCA overdosage. The former hypothesis would seem to be the more tenable.

Summary. The blood pressure of rats rendered hypertensive by treatment with desoxycorticosterone acetate continues to rise following total nephrectomy. Hypertension of this type is not dependent upon a renal mechanism for its maintenance and it is not a consequence of renal vascular damage which is a later development. The relationship of the adrenal cortex to certain forms of clinical hypertension is discussed.

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17302. Relation of Fat Deficiency Symptoms to the Polyunsaturated Fatty Acid Content of the Tissues of the Mature Rat.*

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In a previous study[1] on the appearance of essential fatty acid deficiency symptoms in the mature rat, only the external symptoms were considered. As one of the possible explanations for the ultimate disappearance of the scaly paws, it was suggested that synthesis of at least a limited amount of an essential fatty acid might take place within the animal itself. In order to check this possibility it was decided to examine the tissues for the amount of polyunsaturated fatty acids present at the various stages of development and disappearance of the symptoms.

Experimental and results. The kind of rats employed and the general procedures used in this experiment were similar to those described previously.[1] Fat-free diet A[1] was fed throughout the entire experiment. The fat soluble vitamins were suspended in propylene glycol and were fed once weekly. At the end of the depletion period, during which the intake of food was limited to approximately 5 g per rat per day, 9 out of 39 rats were killed and the entire body fat was extracted for analysis, with special care to prevent any change in the fat, *e.g.*, a low temperature (40-50°C) was maintained in the vacuum oven during the drying of tissues, and a nitrogen ebullition tube was used in all concentration operations.

The thirty remaining depleted rats were given food *ad libitum,* and 35 days later, when all showed symptoms of fatty acid deficiency, 10 rats were killed for analysis. After 71 days on the *ad libitum* regimen, at which time most of the symptoms had largely, though not completely, disappeared, 10 more rats were killed and their fat analyzed as representative of the third stage. Two groups of 5 rats each were killed on the 88th day (stage 3A); those in one group had been supplemented with 2 drops (approximately 100 mg) of ethyl linoleate daily during the last 23 days.

The polyunsaturated constituents of the body lipids were determined by the modified method of Brice *et al.*,[2,3] using alkali isomerization followed by ultraviolet absorption measurements with the Beckman spectrophotometer. It should be mentioned that Brice and coworkers estimated that the probable error of the results is roughly ± 10% of the quantity actually present when that quantity is about 10% of the total fat; and about ± 25% when unsaturated acid measured constitutes only 1% of the fat analyzed. The results of the analysis which represent the average of 4 separate determinations are presented in Table I.

On the basis of these data, certain trends may be observed. At the end of the depletion period (stage 1) the total amount of fat in the body is low, but the percentages of linoleic and arachidonic acid in the fat are very high; the amount of arachidonic acid per rat is also high.

In the second stage, when the deficiency symptoms appeared, a sharp reduction in the amount of linoleic acid and arachidonic acid was noted. The linolenic, which was not found in the first stage, appeared in a small amount at this stage. It is interesting to note that with the small reduction of arachidonic acid there was a steady increase in the

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TABLE I.
Unsaturated Fatty Acids in the Bodies of Rats on Fat Deficient Diet and of Those Supplemented with Ethyl Linoleate.

		No. of rats	Avg wt., g	Avg lipid content/ rat, g	Linoleic*		Linolenic*		Arachidonic*	
					%	g/rat	%	g/rat	%	g/rat
Stage 1	Depletion	9	131	3.17	2.18	.068	0	0	5.76	.183
Stage 2	Appearance of symptoms	10	215	21.1	.38	.080	.37	.078	.686	.145
Stage 3	Recovery	10	276	38.4	.58	.222	.36	.138	.332	.127
Stage 3A	17 days later	5	282	40.4	.65	.252	.44	.178	.299	.121
Stage 3A +	linoleate	5	323	66.6	1.71	1.139	.21	.140	.388	.258

* As calculated by the method of Brice *et al.*[2,3]

amounts of linoleic and linolenic acids as the rats were maintained on the fat free diet beyond their recovery stage.

The supplementation of ethyl linoleate resulted in considerable increase in total fat and the levels of both linoleate and arachidonate present in the fat. At the same time the level of linoleneate declined.

Discussion. It is interesting that the appearance of the essential fatty acid deficiency symptoms coincides with a sharp reduction in the levels of linoleic and arachidonic acids. The accuracy of the zero value for the linolenic acid at the first stage is doubtful. The dependent nature of the simultaneous analysis for the three acids and the high level of arachidonic could have obscured the small amounts of linolenic which might have been present. The appearance of some linolenic in the second stage, when the levels of both arachidonic and linoleic declined sharply, seems to support this doubt.

The increase in the total amounts of linoleic and linolenic at the period when the deficiency symptoms had largely disappeared (3rd stage) is further indicated in the unsupplemented group of 5 rats which were killed 17 days later. Considering the fact that the diet of the rats was devoid of these acids, these findings support the explanation suggested in the previous paper,[1] namely that at least a limited amount of essential fatty acids is synthesized in the body of the mature rat. At these levels the increase of linoleic and linolenic acids only is observed. It is obvious, however, from the arachidonic acid level of the group supplemented with linoleate, that the level of arachidonate also increases in the body at least when the linoleate is administered orally or when it reaches a certain level in the tissues.

Sinclair[4,5] concluded that the essential fatty acids including arachidonic are probably synthesized by the rat on a fat free diet, but not to a sufficient extent to fulfill the requirements. Investigators at the Massachusetts Institute of Technology[6] fed rats a synthetic diet containing tri-di-deuterioiso-olein for 42 days. They analyzed the fatty acids from the carcasses and found that the arachidonic acid increased as the experiment continued, suggesting that arachidonic was synthesized by the rat. This was further supported by their findings that deuterium was present in the arachidonic acid molecule, for it indicated that the arachidonic was synthesized from the deuteriumated iso-oleic acid in the diet.

In the present study the supplemental feeding of linoleate, which raised the levels and amounts of linoleate and arachidonate in the bodies, appears to have lowered the levels of linoleneate. These findings support similar observations made by Rieckehoff *et al.*[7] They found that the trienoic acid content of heart fatty acids decreased upon supplementation with corn oil. They also found that when the fat deficient diet was supplemented with corn oil a considerable deposition of arachidonic or tetraenoic acid took place, indicating

[4] Sinclair, R. G., *J. Biol. Chem.*, 1932, **96**, 103.

[5] Sinclair, R. G., *J. Biol. Chem.*, 1936, **114**, xciv.

[6] Anonymous, Report of Progress in Research III, Nutr. Biochem. Lab., Department of Food Technology, Massachusetts Institute of Technology, 1948, pp. 7-8.

[7] Rieckehoff, I. G., Holman, R. T., and Burr, G. O., *Arch. Biochem.*, 1949, **20**, 331.

to them that this acid could be synthesized from linoleate. Nunn and Smedley-Maclean[8] also observed that supplementation with linoleic or with linolenic acids resulted in the production of arachidonate.

While this work was in progress, Rieckehoff, Holman and Burr[7] reported on the effect of dietary fat on polyethenoid fatty acids of rat tissues. One of their conclusions was that the deposition of polyunsaturated fatty acids takes place primarily in the phospholipid fraction with very little change in the neutral fat. They also found that the effect of the diet on the occurrence of the unsaturated fatty acids is considerably greater in some organs than in others, and that the polyunsaturated acids are particularly low in the skin and depot fat. Smedley-Maclean and Nunn[9] also found small amounts of arachidonate in the phospholipids of liver and muscle of fat deficient rats, but none in their neutral fat.

On the basis of these observations it becomes evident that the phospholipid fraction from the various organs (especially the heart, liver, brain and kidney) should be isolated and analyzed separately for better evaluation of the changes which take place in the essential fatty acid content of the rat. Otherwise, the small amounts of these acids are diluted in the relatively large quantities of fat extracted from the entire carcass, and the changes become less evident.

Summary. 1. Caloric restriction, causing severe emaciation, followed by *ad libitum* feeding on the same fat-deficient diet, precipitated symptoms of fat-deficiency in mature rats. When the rats, after depletion, were maintained on the fat-free diet *ad libitum* for sufficiently long periods, spontaneous recovery was observed.

2. The tissues of these rats were analyzed for linoleic, linolenic and arachidonic acids by the spectrophotometric method. The concentrations of linoleic and arachidonic acids in the body fat were found to vary with the appearance of the symptoms; it was high at the end of the depletion period, and low at the stage when fat deficiency symptoms were present. The higher levels of linoleic and linolenic acids at the time of recovery, after a long period on the fat-free diet, are considered as further evidence for the synthesis of essential fatty acids in the mature rat.

3. Supplementation of ethyl linoleate increased the level of arachidonate in the body fat.

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[8] Nunn, L. C. A., and Smedley-Maclean, I., *Biochem. J.*, 1938, **32**, 2178.

[9] Smedley-Maclean, I., and Nunn, L. C. A., *Biochem. J.*, 1940, **34**, 884.

17303. The Blocking Effect of Nembutal on the Ovulatory Discharge of Gonadotrophin in the Cyclic Rat.

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(Introduced by Duncan C. Hetherington.)
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In a preceding investigation[1] it was indicated that in 4-day cyclic rats of the Vanderbilt strain, under our colony conditions, neurohumoral stimulation of the hypophysis occurs with great uniformity on the day of proestrus between 2 P.M. and 4 P.M., inciting ovulatory discharge of luteinizing hormone. Administration of intravenous dibenamine (N,N-dibenzyl-β-chloroethyl amine) or subcutaneous atropine at 2 P.M. or earlier characteristically blocks ovulation. Injection of either agent at 4 P.M. or later, however, rarely interferes. We have now found essentially identical time relationships with Nembutal (pentobarbital sodium).

Two dose levels of the barbiturate were

[1] Everett, J. W., Sawyer, C. H., and Markee, J. E., *Endocrinology*, 1949, **44**, 234.

TABLE I.
Blocking of Ovulation by Nembutal When Injected at a Critical Hour (2 P.M.) on the Day of Proestrus.

Exp. series	No. of rats	Nembutal treatment		Autopsy interval post-inj.	Results		
		Hr inj.	Dose, mg/kg		Ovulation prevented	Partial interference	Fully ovulated
I	2	2 P.M.	50	19 hr	2	0	0
	2	"	30	20 hr	2	0	0
II	11	"	30	2-4 days	11*	0	0
III	2	"	50	2-3 "	2†	0	0
	19	"	30	2-3 "	19†	0	0
IV	5	4 P.M.	50	18 hr	1	1	3
	5	"	30	18 hr	1	0	4
Totals	36	2 P.M.	30-50		36	0	0
	10	4 P.M.	30-50		2	1	7

* Ovulation was prevented throughout by certain procedures on the second and third days. (See text).

† Ovulated before termination of experiment, but age of corpora lutea at autopsy indicates clearly that ovulation was retarded one day by the initial treatment.

employed: 30 and 50 mg/kg body weight, in single intraperitoneal injections unless otherwise specified. The concentration was 60 mg/ml in a medium containing 20% propylene glycol and 10% alcohol. The lower dose, the more generally desirable, usually produces moderate anesthesia for an hour or more, with somnolence and other signs persisting in varying degrees for several hours.

The animals were adult virgin females of the Vanderbilt substrain Va, as used in the earlier work.[1,2] In each rat the existence of regular 4-day cycles had been established by the vaginal smear method. In such individuals the expected ovulation time is from 1:00 to 2:30 A.M. during the night following proestrus,[2] 9 to 11 hours after the critical period during which neurohumoral stimulation of the hypophysis presumably occurs (see above).

At the end of each experiment the animal was killed with illuminating gas. Tubal ova were looked for in the excised ampullae compressed in physiological saline between a slide and cover slip.[2] The ovaries were fixed in Zenker's fluid, serially sectioned at 10 μ and stained by a modified Mallory tri-acid technic.

In all rats injected with Nembutal at 2 P.M. during proestrus, ovulation was prevented from occurring at the normal time (Table I). This was proven in series I by the presence of unruptured follicles on the day after treatment. In series II the proof was less direct but no less certain. Although autopsy was delayed 2 to 4 days after the initial treatment, hypophyseal stimulation on intervening days was prevented by either atropine (at 2 P.M. on the second day) or by prolonged Nembutal sedation (repeated injections beginning at 2 P.M. each day). Persistent follicles and complete absence of recent corpora lutea in these 11 rats at the termination of experiment demonstrated that the initial brief action of Nembutal during proestrus in the 2-4 P.M. interval postponed the (potential) activation of the hypophysis for a full 24 hours. In series III various modifications of treatment on the second and third days allowed stimulation of the hypophysis on one or the other of these days, as judged from the histological appearance of the new corpora lutea. Here again, the initial treatment during proestrus had delayed stimulation for a full day.

Quite differently, in series IV, when injections were made at 4 P.M. during proestrus, little interference with gonadotrophin release occurred. The high proportion of animals fully ovulating overnight compares with that

2 Everett, J. W., *Endocrinology*, 1948, **43**, 389.

found after other blocking agents at this hour.[1]

The blocking capacity of Nembutal *per se*, in single dose (30 mg/kg), can endure through most of 4 hours at least, since 2 proestrous rats injected at noon were "blocked." The effect does not last much longer, however, for 2 proestrous rats injected at 10 A.M. ovulated during the following night. In control experiments, 4 proestrous rats were injected intraperitoneally at 2 P.M. with 0.1 ml of 20% propylene glycol in 10% alcohol. All ovulated overnight.

The fact that Nembutal will prevent ovulation when administered at appropriate hours, furnishes additional strong evidence that neurogenic stimulation of the hypophysis is essential for ovulatory discharge of LH. Postponement of hypophyseal stimulation for a full 24 hours following the brief action of Nembutal during the 2-4 P.M. interval, implies a 24-hour periodicity in some neural (presumably hypothalamic) center which constitutes a part of the LH-release apparatus in this species. Evidence of similar nature was obtained recently in a quite different experiment, based on the 24-hour advancement of ovulation time by progesterone in 5-day cyclic rats.[3,4]

It is theoretically possible to block ovulation day after day by treatment with barbiturates at appropriate hours. We have, in fact, accomplished this in several cases (series II), but the question of precise timing on days following proestrus requires further analysis. Whenever we succeeded, the vaginal smears gave evidence of continued estrogen secretion and, therefore, of continued secretion of gonadotrophin at subovulatory level. This recalls the report of Westman[5] that certain rats were in constant estrus during 21 days of treatment with twice-daily subcutaneous 1-methyl-5,5-ethylphenyl barbituric acid.

Summary. Brief action of Nembutal during certain critical hours on the day of proestrus delays the ovulatory discharge of hypophyseal gonadotrophin for 24 hours. This is evidence of a 24-hour periodicity in the neural mechanism which incites such discharge.

[3] Everett, J. W., *Anat. Rec.*, 1949, **103**, 448.

[4] Everett, J. W., and Sawyer, C. H., unpublished.

[5] Westman, A., *Acta Med. Scand.*, 1947, **128**, Suppl. 196, 111.

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17304. Lethal *Brucella* Infections in White Mice Produced with the Aid of the Mucin Technic.

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Although the mucin technic has been employed in order to lower resistance against many microorganisms, no attempts have hitherto been made to produce lethal infections with *Brucellae* by means of this technic. It was found that the experimental infections produced in mice without the use of the mucin technic are mild, following a retrogressive and chronic course, so that the mouse may be used as a reservoir for keeping these pathogenic organisms alive. Lethal infections of mice have so far not been reported.[1,2] The following experiments were undertaken in order to produce more acute or even lethal infections with the aid of the mucin method. We expected that the fatal outcome of such infections would enable us to determine the virulence of *Brucellae*, the immunizing power

[1] Lustig, A., and Vernoni, G., in *Kolle, Kraus, Uhlenhuth, Handbuch der pathogenen Mikroorganismsn*, 1928, **4**, 511.

[2] *Topley and Wilson's Principles of Immunity*, revised by G. S. Wilson and A. A. Miles, 3rd edition, London, E. Arnolds & Co., 1948, **1**, 828.

TABLE I.

Lethal Effect of Different Strains of *B. abortus* Injected Intra-abdominally Into Mice Together with 0.5 cc of a 10% Mucin Suspension.

Strain	Quantity injected (cc)	No. of animals			Cumulative numbers		Mortality, %
		Under experiment	Died	Survived	Died	Survived	
Local strain	0.5	8	5	3	8	3	72.7
	0.2	8	1	7	3	10	23.1
	0.1	8	2	6	2	16	11.1
	0.05	8	0	8	0	24	0
Swiss strain	0.5	8	7	1	13	1	92.9
	0.2	8	4	4	6	5	54.5
	0.1	8	2	6	2	11	15.4
	0.05	5	0	5	0	16	0
Abortus 19	0.5	8	4	4	8	4	66.7
	0.2	8	4	4	4	8	33.3
	0.1	8	0	8	0	16	0

of vaccines as well as the protective and therapeutic action of sera and chemotherapeutic substances.

Test organisms. Two strains, one of *B. melitensis* and another of *B. abortus*, isolated in this country were employed. In addition to these, 3 laboratory strains of *B. abortus* were employed. One was obtained from the National Type Collection in London and 2 others representing the attenuated "Abortus 19" from the Government Veterinary Laboratories in Tel Aviv. One of these strains, designated as "Swiss strain 19" proved to be richer in S-forms than the other.

Mucin. Mucin in scales manufactured by Burroughs Wellcome, London, was employed. A weighed amount was thoroughly ground in a mortar with glass sand, suspended in saline to give the required concentration and autoclaved. Concentrations of 5.0%, 7.5%, and 10.0% were employed.

Experimental infections. White mice from the laboratory stock served as experimental animals. The mice were 5 weeks old at the time of the tests and averaged 20 g in weight. Preliminary experiments with 48-hour broth cultures showed that 0.1-0.5 cc of these cultures given intra-abdominally did not produce lethal infections, though after sacrificing the animals 5 days after injection, pure cultures of *Brucellae* could be obtained from most organs. The spleens of these animals showed marked enlargement. Another group of animals now received varying quantities of 48-hour broth cultures ranging from 0.005 to 0.5 cc, to which 0.5 cc of mucin in varying concentrations was added. These experiments which were carried out with *B. abortus* as well as with *B. melitensis* showed that a mucin concentration of at least 10% must be employed in order to obtain a lethal effect; 0.5 cc of broth culture produced a 100% mortality and 0.2 cc of broth culture a 50% mortality when injected together with 0.5 cc of a 10% mucin suspension. Mucin suspensions of 5.0% and 7.5% given with the same quantities of broth cultures were not able to produce a lethal effect. No differences were observed between *B. melitensis* and *B. abortus*.

We now examined the lethal effect of different strains of *B. abortus* given under the same conditions. The results of these experiments are summarized in Table I. While 2 of the strains, the local strain and the "Abortus" 19 proved to be relatively avirulent, both exhibiting their LD_{50} at 0.35 cc, the Swiss strain proved to be more virulent for mice, if injected along with mucin, having a LD_{50} at 0.19 cc.

The mice which succumbed to the infection yielded pure cultures of the infective strains from their organs. Death generally occurred after 1-2 days, though with small doses such as 0.1 cc it sometimes occurred after 4-8 days. The shorter the period between the injection and death the less was the spleen enlarged.

The surviving animals of the different ex-

periments were killed within a period ranging from the sixth to the 50th day after injection. The organs of the infected animals were cultured on agar plates as well as in broth tubes. In many cases the agar cultures yielded negative results, while after incubation of the broth cultures for 7 days positive results were obtained. The microorganisms isolated from these cultures were identified as *Brucellae* by agglutination with specific antisera. The following organs were examined: heart, lungs, liver, spleen, kidneys and genital glands. Seventy-six mice which were dissected up to the 28th day after injection proved to harbor *Brucellae* in all or in several organs. Fifteen out of 20 mice which were dissected between the 30th and the 50th day after the onset of the infection proved to harbor *Brucellae*, while only 5 yielded sterile cultures. The organs of 145 mice were cultured after death took place or when the mice were sacrificed. The positive results obtained from the different organs were as follows: Heart 131, lungs 126, liver 133, spleen 136, kidneys 136, genital organs 127. These figures prove that significant differences in the infection rate of the different organs do not exist, but only that foci of infection are equally distributed over the different organ systems of the infected animal.

Summary. *B. melitensis* and *B. abortus* did not produce lethal infections in mice even when 0.5 cc of broth cultures were injected intraabdominally. On the other hand lethal infections were produced if the broth cultures were administered together with 0.5 cc of a 10% mucin suspension. The LD_{50} for *B. melitensis* was 0.2 cc, for 2 strains of *B. abortus* 0.35 cc, and for a third strain of *B. abortus* 0.2 cc. In most of the surviving animals chronic infections were noted, if the dissections were performed up to the 50th day after the onset of the infection.

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17305. Absorption of Subtilin in the Rabbit.

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Subtilin,[1,2] an antibiotic active *in vitro* against Gram-positive bacteria[3] and *M. tuberculosis*,[3-6] has also been found active *in vivo* against the more sensitive pathogenic bacteria. For example, Salle and Jann injected subtilin intraperitoneally into mice infected with pneumococcus Type III,[7] *Streptococcus pyogenes*,[8] or *Staphylococcus aureus*,[9] and into guinea pigs subjected to experimental anthrax infections.[10] Prophylactic and therapeutic effects, depending on the relations of infection and dosage times, were striking.

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1 Garibaldi, J. A., and Feeney, R. E., *Ind. Eng. Chem.*, 1949, **41**, 432.

2 Fevold, H. L., Dimick, K. P., and Klose, A. A., *Arch. Biochem.*, 1948, **18**, 27.

3 Salle, A. J., and Jann, G. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 60.

4 Wong, S. C., Hambly, A. S., Jr., and Anderson, H. H., *J. Lab. Clin. Med.*, 1947, **32**, 837.

5 Knight, V., Shultz, S., and DuBois, R., Proc. 48th General Meeting Soc. Am. Bact., Minneapolis, Minn., May, 1948, p. 84.

6 Knight, V., and Tompsett, R., *J. Clin. Invest.*, 1948, **27**, 544.

7 Salle, A. J., and Jann, G. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 40.

8 Salle, A. J., and Jann, G. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 519.

9 Salle, A. J., Presentation at the Conference on Antibiotic Research, Washington, D. C., January 31 and February 1, 1947, under the auspices of the Antibiotics Study Section of the National Institute of Health.

10 Salle, A. J., and Jann, G. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 41.

These findings have been confirmed with respect to pneumococcal and streptococcal infections by Knight and colleagues[5,6] and with respect to protection against *Streptococcus hemolyticus* by Hobby.[11] On the other hand, in spite of *in vitro* potency, subtilin has been found ineffective against syphilitic infections in rabbits[12] and against *M. tuberculosis* infections in hamsters[13] and in mice.[5,6,14] Steenken and Wolinsky[15] found subtilin without effect on tuberculosis infections in guinea pigs, whereas Salle and Jann[16] have reported both positive and negative results with different lots of subtilin. Farber, Eagle, Anderson, and Gorman[17] found suggestive evidence of therapeutic activity from the topical application of subtilin on tuberculous laryngeal lesions.

The present work was undertaken to determine the absorption of subtilin and of methyl esters of subtilin[18] when administered by various routes and to note any pharmacological effects, as an indication of the value and the feasibility of more extensive clinical trials. The activity of these esters against *M. tuberculosis in vitro* is at least as high as that of subtilin.[19]

Methods. Thirty-one rabbits were used in 44 experiments. The animals used more than once had shown little or no absorption at the time of the original experiment and had not been used for at least a month. Except for treatments by slow infusion or injection into the ligated colon, the animals were not anesthetized. Anesthesia, when used, was with sodium pentobarbital. Initially, 40 mg/kg intravenously was used, with 10 mg/kg supplements as needed to maintain a light anesthesia. Intravenous injections of subtilin were made into the marginal ear vein, subcutaneous injections were divided between the two sides of the animal so as to insure a larger surface for absorption. Intramuscular injections were divided likewise and were made into the muscles of the thigh.

The subtilin was in nearly all instances from one composited batch of highly potent material prepared by methods described elsewhere.[1,2] Although electrophoretic analysis, fractional dialysis and salt fractionation[2] indicated that our subtilin was homogeneous, investigations at the research laboratories of Merck & Co., Rahway, N. J., by countercurrent distribution showed that our samples were probably about 90% pure. The methyl esters were prepared[18] from another batch of equally pure subtilin. Two lots were used: the first (No. 17ME-242) contained 5.9 equivalents of methoxyl per 10^4 g and was characterized by markedly enhanced bacteristatic potency and 4-fold increase in solubility under physiological conditions. The second (No. 37ME-242) contained 11.9 equivalents of methoxyl per 10^4 g and was characterized by substantially unchanged bacteristatic potency but 30-fold increase in solubility under physiological conditions. For intravenous administration, the solvent was 5% glucose; when 10% subtilin solutions were used for administration by other routes, the subtilin was dissolved in distilled water. Blood samples for subtilin assay were obtained by cardiac puncture. Urine, when desired, was obtained by catheter with washing of the bladder so as to assure complete collection.

Subtilin and the methyl esters of subtilin were determined by the cup-plate method. The test organisms used previously[20] for the

11 Hobby, G., personal communication.

12 Eagle, H., Musselman, A. D., and Fleischman, R., *J. Bact.*, 1948, **55**, 347.

13 Anderson, H. H., and Wong, S. C., *Tuberculology*, 1946, **8**, 77.

14 Rake, G. W., personal communication.

15 Steenken, W., Jr., and Wolinsky, E., *J. Bact.*, 1949, **57**, 453.

16 Salle, A. J., and Jann, G. J., Presentation at the Second National Symposium on Recent Advances in Antibiotics Research, Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section of the National Institute of Health.

17 Farber, S. M., Eagle, H. R., Anderson, H. H., and Gorman, R. D., *J. Lab. and Clin. Med.*, 1948, **33**, 799.

18 Carson, J. F., Jansen, E. F., and Lewis, J. C., *J. Am. Chem. Soc.*, in press.

19 Chin, Y. C., *Fed. Proc.*, 1948, **7**, 211, and unpublished data.

20 Lewis, J. C., Humphreys, E. M., Thompson, P. A., Dimick, K. P., Benedict, R. G., Langlykke, A. F., and Lightbody, H. D., *Arch. Biochem.*, 1947, **14**, 437.

turbidimetric assay of subtilin were much too insensitive by the cup-plate technic. This insensitivity proved particularly troublesome for the methyl esters of subtilin, which had much lower potencies than subtilin by cup-plate or agar-streak methods in contrast to their behavior in broth. The cause of this anomalous behavior is not known. A bacterium tentatively identified as *Sarcina lutea* was used as the test organism for subtilin. The test medium was that previously used[20] for the turbidimetric assay of subtilin with *Micrococcus conglomeratus*. Incubation for 20 hours at 35° gave an approximately 20-mm zone of inhibition when a solution containing 1 ppm of subtilin was placed in the cup. An unidentified Gram-negative diplococcus (C-7) isolated from chicken feces was chosen as the most sensitive organism available for the assay of methyl esters of subtilin. Two ppm of either ester gave an approximately 20-mm zone on nutrient agar containing 5% NaCl after 22 to 48 hours' incubation at 35°C.

In all assays the antibiotic under test was dissolved in an aliquot of whole citrated rabbit blood to provide a standard response. Control blood samples taken from animals never treated previously gave no inhibition of the test organisms. Citrated blood samples from treated animals and aqueous dilutions of this blood were pipetted directly into assay cups. Dilutions were not necessary for blood samples from animals treated with the subtilin esters. All assay results are expressed in terms of the weight of the particular type of subtilin under test, since the relative potencies of the three materials varied markedly with the experimental conditions.

Results. Subtilin. Intravenous injection gave a satisfactory blood level, but the level was transitory and the procedure was accompanied by some danger. In a single experiment, 50 mg/kg caused death in 2 minutes, and 10 mg/kg did likewise in 1 of 5 trials. The concentration of subtilin in the blood of the other 4 animals was 100-200 ppm 5 minutes after the injection, falling to 10-30 ppm in 2 hours and to zero in 24 hours. Up to 7% of the injected subtilin was found in the 2-hour urine.

Subcutaneous and intramuscular injection of 100 mg/kg can be considered together. Blood levels ranging from a trace up to 1.4 ppm were found. A persistent lump was present at the injection site after subcutaneous injections, and a sterile abscess was present in muscles 2 months after subtilin administration. There was no antibiotic activity in an extract of this abscess, although an assay of a comparable muscle site 24 hours after injection indicated that the granular material still had considerable activity.

Intraperitoneal injection of 10 to 100 mg/kg led to blood levels of 0 to 1 ppm of subtilin. After 10 mg/kg there was no visible precipitate in the abdomen, but 50 mg/kg was sufficient to produce such a deposit.

Treatment of subtilin by crystalline pepsin or trypsin reduces its antibiotic activity.[21,22] This fact, along with the known low solubility of subtilin in body fluids, would suggest that administration by mouth would be ineffective. Nevertheless, 1 g/kg was given to one rabbit *per os*. The highest blood level noted was 0.1 ppm. After 5 hours, of the 4 grams originally given, only 2 μg were found in the urine, 15 mg in the intestine, and 0.5 g in the stomach. Two anesthetized animals were given 100 mg of subtilin/kg per rectum, with ligation of the gut near the anus to prevent loss. The solution was 1% of subtilin in water, and the volume was great enough to spread throughout the colon. From 2 to 4 ppm of subtilin were found in the blood stream, and autopsy 2 hours after administration showed subtilin deposits in the colon.

It is conceivable that a slow subcutaneous infusion of a dilute solution would allow the subtilin to be absorbed rather than precipitated. Concentrations of 0.05 to 0.5% in 5% glucose were infused in 3 unanesthetized animals under the skin of the back at rates of 1.7 to 27 mg/kg/hr for 6 hr. The volume of solution was approximately 5 cc/kg/hr. Some absorption did occur, as 27 mg of subtilin was found in the 24-hour urine sample in one

21 Stansly, P. G., and Ananenko, N. H., *Arch. Biochem.*, 1947, **15**, 473.

22 Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., *Arch. Biochem.*, 1947, **15**, 1.

instance and 4 mg in another; however, absorption was so slow that 6 ppm of subtilin was the maximum found in the blood.

The preceding experiments have indicated that little subtilin gets into the blood stream unless it is placed there directly. In the case of a single injection, there is some danger and activity is short-lived. A slow, intravenous infusion might give satisfactory blood levels without seriously embarrassing the animal. Three anesthetized rabbits were used, with infusion into the jugular or femoral veins of 0.25% subtilin in 5% glucose. One animal received 20 mg/kg/hr for 2 hours, 60 mg/kg/hr for 1 hour and 150 mg/kg/hr for 30 minutes until death. At the end of the third hour the blood level was 450 ppm and 6% of the injected subtilin was found in the urine. No symptoms were observed until sometime after the rate was raised to 60 mg, when tremors in the extremities were seen. Death was apparently of cardiac origin. The second animal was given 20 mg/kg/hr for 4 hours. No symptoms were noted, the animal appearing in good condition at the end of this time. The blood level had risen to 700 ppm.

In these 2 rabbits, the blood was observed for agglutination. We had found previously that there was an *in vitro* clumping of red cells at concentrations of subtilin lower than found in these two animals. No clumping was noted, indicating that *in vivo* actions differed from *in vitro*, or that agglutinated cells had been filtered out in the capillary systems of the body.

The third rabbit receiving intravenous infusion was prepared for records of blood pressure and respiration. Approximately 20 mg/kg/hr for 1½ hours did not modify blood pressure, heart rate or respiratory rate. Raising the infusion rate to 70 mg/kg/hr caused a gradual fall in pulse pressure (as measured with a membrane manometer) and a gradual increase in respiratory rate.

Chin[23] has reported that the LD_{50} of intravenously injected subtilin to mice is 100 mg/kg. This is slightly higher than the approximately 70 mg/kg found by us in a small series of animals, but either value would give a body concentration well above the minimum *in vitro* effective tuberculostatic dose. Death from intravenously administered subtilin is presumably due to embolism since subtilin, which dissolves readily in water, is only slightly soluble in physiological saline[18] or serum.[24] The amount held in solution varies somewhat with the manner of manipulation, being around 0.05 to 0.1 g/100 cc under physiological conditions of temperature and salt concentration. The intraperitoneal LD_{50} was some 2 to 3 times the intravenous LD_{50}. Subcutaneously, mice were able to tolerate more than 3 g/kg of purified subtilin without demonstrable symptoms.[25] The lack of subcutaneous toxicity is likewise explained by the low solubility in body fluids, a deposit of precipitated material being formed at the site of injection.

Subtilin methyl esters. The increased solubility of some of the methylated subtilin preparations, and the increased antibiotic activity of others[18] led to the hope that satisfactory blood levels could be obtained. Twelve animals were used. On 4 of them blood assays were not made. There were no untoward reactions in these or any of the other rabbits receiving the esters. It was found that blood levels of the 8 other animals were slightly higher after intravenous, subcutaneous and intramuscular injection of the more soluble ester than the levels found after administration of unmodified subtilin. The levels (up to 2.4 ppm after intramuscular administration) were not great enough to warrant further study. The ester with increased activity was absorbed so poorly that it had an unimportant antibiotic effect in the blood.

Several lots of methyl esters of subtilin were tested for approximate intravenous toxicity in mice. The values obtained did not differ markedly from values for unchanged subtilin.

Subtilin-pectin complex. Subtilin, when dissolved with 5 to 8 times its weight of pectin gives a product which is soluble in physiological saline to the extent of 0.5% of subtilin. After some time the complex begins to

23 Chin, Y. C., *Fed. Proc.*, 1947, **6**, 317.

24 Klose, A. A., unpublished data.

25 Wilson, R. H., Lewis, J. C., and Humphreys, E. M., *Fed. Proc.*, 1948, **7**, 266.

break down and subtilin comes out of solution. Dr. Harry S. Owens of this laboratory prepared such a complex for us. It was given intravenously 3 times, intramuscularly and subcutaneously once each. After intravenous injection it disappeared from the blood as rapidly as did unmodified subtilin, and it was as poorly absorbed when given by the other routes. Since it offered no advantages over subtilin and, because of its great viscosity, was very difficult to inject or else required considerable dilution, it was not considered further.

Pectin-treated subtilin. At a recent symposium, Salle and Jann[16] stated that a described treatment of subtilin eliminated abscess formation and that subcutaneously injected material so treated was effective against tuberculosis in guinea pigs. The treatment consisted in dissolving 1.5 g of subtilin in 50 ml of 10% urea solution and mixing with an equal volume of 0.1 g of pectin in distilled water. After standing overnight, the supernatant liquid was used for injection. Following this report, we prepared a subtilin solution in the described manner and injected it subcutaneously into 2 rabbits. The dosage was 100 mg/kg, assuming that none of the subtilin was lost during the manipulations. Assays of periodic blood samples showed blood concentrations similar to those reported earlier in this paper.

The question of abscess formation was studied on mice and guinea pigs. We felt that lack of abscesses might be due to simple dilution. Four groups of mice were used, 6 animals to a group. Each mouse received 1 ml of solution under the skin of the back, as follows: (1) 1.5% subtilin, Lot No. 317, in 5% glucose, (2) 5% subtilin No. 317; (3) the solution prepared as described by Salle and Jann, using subtilin No. 317; (4) a 5% solution of subtilin recovered from (3) by precipitation with 10% NaCl.[2] All animals in groups 2 and 4 and a majority in groups 1 and 3 showed abscess formation, the abscesses being smaller and less apt to ulcerate with the more dilute solutions.

Three guinea pigs were used, each animal receiving subcutaneously the following 4 solutions: (1) 1.5 ml of the solution prepared as described by Salle and Jann; (2) 1.5 ml of 1.5% subtilin No. 317 in 5% glucose; (3) the same *weight* (22 mg) of subtilin in a 10% solution; (4) the same *volume* (1.5 ml) of subtilin in a 10% solution. A large, hard, subcutaneous lump was formed in all 3 animals by solution 4. Solution 3 caused a prompt development of small lumps in 2 animals, and solutions 1 and 2 produced more slowly developing lumps in 1 and 2 animals, respectively. These results indicate that the proposed treatment does not modify the absorption of subtilin nor its tendency to produce abscesses. The decreased reaction was due to the dilution of the material. Since approximately 1/3 of the subtilin was precipitated by the pectin (as estimated by sulphur balance) the concentration of subtilin in the solution prepared according to the method of Salle and Jann was actually about 1%.

Discussion. Consideration of the foregoing results indicates that high systemic levels of subtilin are not obtained readily in the rabbit. The amount absorbed after administration in a variety of ways would not produce bactericidal or even bacteristatic levels in the blood stream, except possibly for very sensitive Gram-positive organisms. Furthermore, injection under the skin or into muscle is followed by a lasting deposit which is gradually transformed into an abscess. The poor absorption and the local deposition of precipitated subtilin are caused by the low solubility of subtilin in physiological fluids. It is possible to obtain substantial blood levels in the rabbit only by intravenous administration. If this is done by single injection there is some danger, due again to low solubility causing precipitation in the blood stream, and furthermore, the blood levels are not maintained. The one method of administration which, within the limits of the above experiments, would give a satisfactory blood level with no ill effects to the animal is a slow intravenous infusion.

It is conceivable that results reported here would differ with other species. In the introduction it was noted that certain infections in mice and guinea pigs could be controlled, and we find mice may be killed by intraperitoneal injections of subtilin. However, the

difference is probably only one of degree. In the mouse, as in the rabbit, a subcutaneous deposit is formed, and in the former animal an abscess is formed at the site of injection. Since the lack of absorption and the manifestations of toxicity are dependent on the physical properties of subtilin in physiological fluids, it seems unlikely that a species difference would be marked.

Such modifications of subtilin as have been tried to date have not greatly modified the physiological absorption or bacteristatic level, even though the products have had somewhat greater *in vitro* antibiotic activity or greatly increased solubility under physiological conditions.

Although the results in this paper indicate that high systemic levels of subtilin are not practical, it is quite possible that subtilin would be beneficial when topical application is indicated. We have observed no reactions which would contraindicate local application.

Conclusions. The antibiotic, subtilin, has been administered to rabbits in a variety of ways, with analyses of blood to determine absorption. The bioassay procedures are described.

Because of precipitation in physiological fluids, injection into subcutaneous tissue, muscle or peritoneal cavity is an ineffective way of reaching bacteristatic levels of subtilin in the blood stream. Administration by mouth or per rectum is likewise unsatisfactory.

A single injection intravenously is accompanied by some danger and gives a blood level of subtilin which is not maintained. A satisfactory level can be maintained, at least for 4 hours, by slow intravenous infusion, without apparent harm to the animal.

A subtilin-pectin complex, which temporarily allows more subtilin to be soluble in physiological saline, a methyl ester of subtilin which is likewise more soluble and which possesses greater antibiotic activity, and a second methyl ester which is much more soluble, are all absorbed in amounts similar to that of unmodified subtilin.

No observations were made which would contraindicate the topical application of subtilin.

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17306. The Antidiuretic Action of Relaxin-Containing Preparations.*

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The phenomenon of water retention in the female during pregnancy has received a great deal of attention from many investigators. It has been known for some time that both an extract of the posterior pituitary and desoxycorticosterone acetate have antidiuretic action. In addition, Thorn, Nelson, and Thorn[1] have shown that the sex steroids can also produce water retention in the dog. Nevertheless a suitable explanation for the shift in water balance during pregnancy is still lacking. In the present report data are presented to show that extracts from the ovaries of pregnant sows and blood serum of pregnant rabbits, prepared for relaxative activity on the symphysis pubis of the guinea pig, possess an antidiuretic action in the rabbit.

Several different extracts of relaxin were used in these experiments. Preparation J-46 was prepared from unselected ovaries† of the sow according to the method of Albert,

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, to Professor Frederick L. Hisaw.

1 Thorn, G. W., Nelson, K. R., and Thorn, D. W., *Endocrinol.*, 1938, **22**, 155.

† Unselected ovaries were obtained from both pregnant and non-pregnant sows.

Money, and Zarrow[2] and showed an activity of 30 guinea pig units (G.P.U.) per mg dry weight in the symphyseal relaxation test (Abramowitz *et al.*).[3] Two extracts, PS-1‡ and OR-24§ were prepared from the ovaries of pregnant sows. Pregnant rabbit serum was obtained from rabbits between the 25th and 28th days of pregnancy, and concentrated with alcohol and acetone (Albert and Money).[4] In addition, control extracts were prepared from beef heart and blood serum of male rabbits. Extensive tests on the relaxin preparation J-46 showed that it contained no estrogenic or progestational activity.

All tests for antidiuretic action were carried out in adult female rabbits of the New Zealand strain weighing from 4.0 to 5.5 kg. The rabbits were placed in a metabolism cage and food and water supplied *ad libitum*. In addition the animals received lettuce thrice weekly. Prior to the test, 24 hour values were obtained for the urine output and water intake for about 2 weeks. Only those rabbits were used that showed a fairly constant output of urine during this period. Injections of the test substances were given subcutaneously 3 times a day for 3 days. The antidiuresis occurred usually within 24 hours after the injections were started and definitely by 48 hours and the response was considered positive if the urine output was decreased by 50% or more and maintained at this level for at least the duration of the treatment.

A sample of the data obtained may be seen in Fig. 1 to 4. It will be noted that the injection of either 10 ml of saline thrice daily for 3 days or a beef heart extract had no effect on the urine output. However, the injection of relaxin (J-46) at a dose level of 1000 G.P.U. 3 times daily decreased the urine output from 180 ml to approximately 27 ml. The same type of response was also obtained with 250 G.P.U. of relaxin injected in the same manner. However, 100 G.P.U. of relaxin gave no effect.

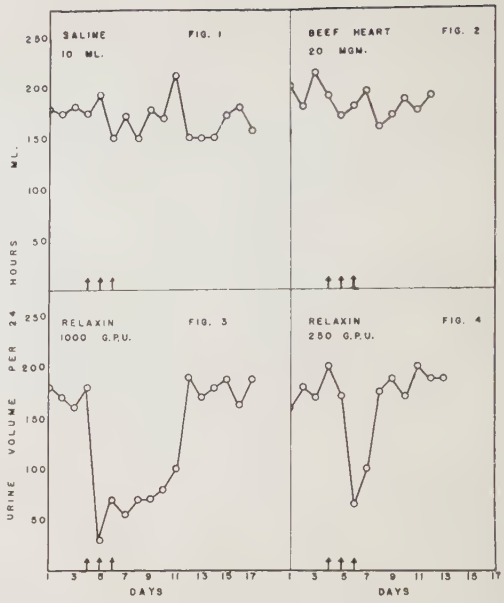

FIG. 1-4.

Twenty-four hour volume of urine in ml plotted against days. Arrows represent days of injection.

FIG. 1. Lack of effect on urine output after treatment with 10 ml of saline injected 3 times daily for 3 days.

FIG. 2. Lack of effect of 20 mg equivalent of beef heart extract injected 3 times daily for 3 days.

FIG. 3. Marked antidiuretic response obtained with 1000 G.P.U. of relaxin (J-46) injected 3 times daily for 3 days.

FIG. 4. Antidiuretic response obtained with 250 G.P.U. of relaxin (J-46) injected 3 times daily for 3 days.

Thus far we have examined a number of preparations for antidiuretic action and have found that relaxin obtained from the ovaries of pregnant sows, nonselected ovaries[3] and pregnant rabbit serum possesses the ability to cause urine retention (Table I). In addition to the controls mentioned above the injection of blood serum from male rabbits also gave negative results. The minimum effective dose of relaxin for the antidiuretic effect was 250 G.P.U. for our preparations and 50 G.P.U. for the Maltine extract. This discrepancy may be explained by the fact that

[2] Albert, A., Money, W. L., and Zarrow, M. X., *Endocrinol.*, 1947, **40**, 370.

[3] Abramowitz, A. A., Money, W. L., Zarrow, M. X., Talmage, R. V. N., Kleinholz, L. H., and Hisaw, F. L., *Endocrinol.*, 1944, **34**, 103.

‡ Obtained through the courtesy of Dr. Edward H. Frieden, Biological Laboratories, Harvard University.

§ Obtained through the courtesy of Dr. Robert L. Kroc, Maltine Company, Morris Plains, N. J.

[4] Albert, A., and Money, W. L., *Endocrinol.*, 1946, **38**, 56.

TABLE I.
Antidiuretic Effect of Relaxin Preparations in the Rabbit.

Relaxin	Dose in G.P.U.*	Source of relaxin	No. of tests	Response
J-46	3000	Ovaries of sows	3	Positive
"	1000	" " "	4	"
"	500	" " "	4	"
"	250	" " "	4	"
"	100	" " "	2	Negative
PS-1	500	Ovaries of pregnant sows	1	Positive
OR-24	1000	Ovaries of pregnant sows	1	Positive
"	500	" " " "	1	"
"	250	" " " "	1	"
"	100	" " " "	1	"
"	50	" " " "	2	"
"	25	" " " "	1	Negative
PR-1	500	Blood of pregnant rabbits	1	Positive
Control	10 ml	Saline	3	Negative
"	1 ml	"	1	"
"	2 ml	"	2	"
"	20 mg	Beef heart	2	"
"	50 ml †	Blood of male rabbits	2	"

* Represents the individual dose that was injected 3 times daily for 3 days.
† The equivalent of 50 ml of blood was injected 3 times daily.

the Maltine unit appears to be approximately 4 times greater than our unit. Thus it would seem that the antidiuretic activity parallels the relaxin activity in extracts prepared in two different laboratories. Furthermore, the presence of the antidiuretic activity in relaxin preparations obtained from such diverse sources as the ovary of the sow and the blood of pregnant rabbits appears to be highly significant. While the evidence is as yet insufficient, there is this striking correlation between the content of relaxin in the preparation and the ability to induce water retention. It may also be pointed out that this new antidiuretic factor is not identical with the posterior pituitary hormone. Donaldson[5] has shown that the latter is dialyzable whereas relaxin and the antidiuretic factor are non-dialyzable.[3] The probability that relaxin is responsible for water retention is also supported by the fact that the blood serum of pregnant rabbits produces both relaxation of the symphysis pubis and water retention whereas the blood serum of male rabbits is without these effects.

The possible identity of the antidiuretic action with relaxin and the fact that the latter substance is found primarily in high concentrations during pregnancy,[6-8] leads to speculation as to whether relaxin may be concerned with the shift in water balance during gestation.

Summary. Relaxin containing extracts of the ovaries of pregnant sows and of the blood of pregnant rabbits possess an antidiuretic action in the rabbit. Some evidence is presented to indicate that the antidiuretic activity parallels the relaxin activity of the preparations used in the present study.

5 Donaldson, W., *J. Clin. Invest.*, 1947, **26**, 1023.

6 Marder, S. N., and Money, W. L., *Endocrinol.*, 1944, **34**, 115.

7 Zarrow, M. X., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 488.

8 Hisaw, F. L., and Zarrow, M. X., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 395.

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17307. A Chemical Method for the Detection of Virus Infection of the Chick Embryo.

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Chemical or physical differences in the allantoic fluid of normal and virus-infected chick embryos have been emphasized by few investigators. McLean *et al.*[1] mentioned differences in the pH of normal and influenza virus-infected allantoic fluids. Parodi and his collaborators[2] reported a slower decline in pH and an increase in volume of the allantoic fluid from embryos infected with influenza virus. In studies with their common cold virus (MR-1) Atlas and Hottle[3] noted high absorption peaks with dialyzed infected allantoic fluid which were attributed to protein. Such peaks were sometimes observed with normal fluid. In view of such observations, it appeared possible that there might be sufficient chemical difference in virus-infected and normal allantoic fluid to permit the development of a chemical test for virus infection of the chick embryo.

Early in the course of a systematic investigation of the properties of infected and normal allantoic fluid, it was discovered that allantoic fluid from embryos infected with influenza virus contained appreciably greater quantities of protein. Accordingly, a simple quantitative method for the determination of protein in allantoic fluid was devised and subsequenty utilized in studies of infection of the allantoic sac with various viruses.

Materials and methods. Allantoic fluid. Allantoic fluids used in turbidity determinations were carefully harvested from 10-12-day-old White Leghorn embryos previously chilled for 12-18 hours at 4°C. Grossly bloody fluids and those inadvertently contaminated by yolk were discarded. Groups of 5-6 embryos were employed.

Viruses. The PR8 and Lee strains of influenza virus and a strain of Newcastle disease virus adapted to the allantoic sac by serial passage were used. The Habel strain of mumps virus, adapted in this laboratory to the allantoic sac, was also utilized. Semliki Forest virus (SFV) in the form of desiccated mouse brain (110th passage) was obtained through the courtesy of Dr. K. C. Smithburn, who had demonstrated[4] rapid multiplication of the virus in the chick embryo. This mouse brain suspension killed embryos within 24-36 hours when injected into the allantoic sac. Subsequent passages were made with allantoic fluid.

Control materials. Allantoic fluids, hereinafter referred to as normal, were obtained from embryos inoculated with normal or heated (65°C for 30 minutes) allantoic fluid diluted 1:10 to 1:1,000 in 0.85% sodium chloride solution buffered to pH 7.2 with phosphate. The same diluent was used for virus inocula.

Turbidity determination. One cc of 10% trichloracetic acid is added to 1 cc of allantoic fluid in a soft glass test tube measuring 100 × 10 mm. Reagents are measured with ordinary serologic pipettes. Two to 5 minutes after the addition of acid, turbidity readings are determined in a Klett-Summerson colorimeter. An adapter for the small test tube and a blue filter (peak transmittance, 420 mμ) are employed. Mixing of reagents is accomplished by inversion of the colorimeter tube. A blank of 10% trichloracetic acid is used for preliminary setting of the zero reading of the colorimeter. In the instrument used in this study a 0.03% suspension of barium sulfate gives a reading of 70. Turbidity is expressed directly in the units comprising the scale of the colorimeter, these units being directly proportional to optical density. Fifteen serial determinations of

[1] McLean, I. W., Jr., Cooper, G. K., Taylor, A. R., Beard, D., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 192.

[2] Parodi, A. S., Lajmanovich, S., Pennimpede, F., and Mittelman, N., *J. Immunol.*, 1948, **58**, 109.

[3] Atlas, L., and Hottle, G., *Science*, 1948, **108**, 743.

[4] Smithburn, K. C., *J. Immunol.*, 1946, **52**, 309.

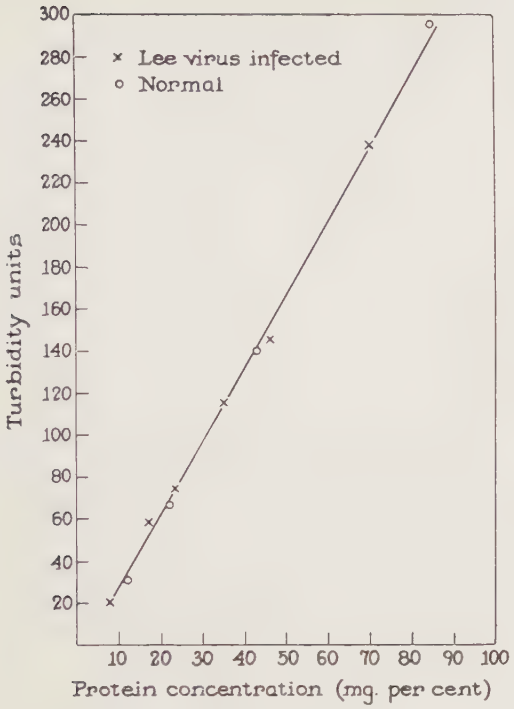

FIG. 1.

Relation between turbidity and protein concentration in allantoic fluid. Turbidity was produced with 10% trichloracetic acid. Protein N_2 was determined by micro-Kjeldahl.

turbidity, using the same allantoic fluid pool, showed an experimental error of ± 2.8% for the procedure as described above.

Experimental. Studies of dialyzed allantoic fluids revealed a substance precipitable by 10% trichloracetic acid to be present in both normal and influenza virus-infected fluids and greatly increased in infected fluids. Similarly, micro-Kjeldahl determinations of total nitrogen demonstrated a higher concentration of nitrogen in fluid from infected embryos. The linear relation of the turbidity with trichloracetic acid and protein concentration of both normal and infected allantoic fluids may be seen in Fig. 1 in which the turbidities of varying dilutions of concentrated dialyzed allantoic fluids are plotted against the protein concentrations of the fluids as determined by the micro-Kjeldahl method. This relationship was found to hold with concentrations up to 500 turbidity units. However, specimens giving readings above 300 were diluted and re-examined because of difficulties experienced in obtained accurate scale readings in the higher range. A similar straight line relationship between serum protein concentration and turbidity produced by trichloracetic acid has been established within certain limits of concentration by Chow *et al.*[5]

Further study of the acid-precipitable substance in allantoic fluid has indicated its protein nature. Dialysis in cellophane against 0.85% saline did not reduce the concentration of the substance. The characteristic biuret, xanthoproteic, and ninhydrin color reactions were given by dialyzed allantoic fluids. Precipitates were formed in dialyzed and nondialyzed fluids by the addition of 95% ethyl alcohol or ammonium sulfate. The antigenicity of the substance, which is discussed below, is further evidence for its protein nature.

Fractionation of dialyzed allantoic fluids by half and full saturation with ammonium sulfate was performed. The protein concentration of these fractions was then determined by micro-Kjeldahl analysis after redialysis against saline. The albumin-globulin ratios in normal and infected fluids did not

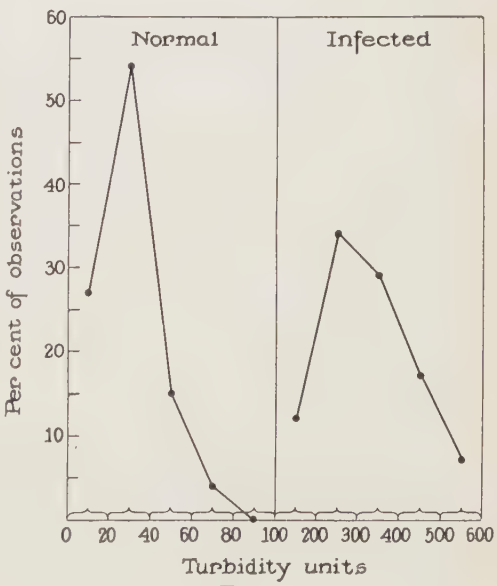

FIG. 2.

Frequency distribution of turbidity values with normal and Lee virus infected allantoic fluids.

5 Chow, B. F., Hall, L., Duffy, B. J., and Alper, C., *J. Lab. and Clin. Med.*, 1948, **33**, 1440.

TABLE I.
Increase in Allantoic Fluid Protein with Various Viruses.

Virus 10-3 dilution inoculated	Time after inoculation, days	No. of eggs	Allantoic fluid turbidity†	
			Range	Mean
Lee	2	41	118-540	316
SFV*	1-1½	17	40-300	119
NDV	2	20	52-260	104
PR8	2	12	30-152	87
MV	4-5	12	56-160	84
Control	2	100	8-75	29
"	4-5	10	21-50	33

* 10-1 to 10-3 dilutions used.
† Turbidity developed with 10% trichloracetic acid.

differ materially, being 7.3/1 in normal and 8/1 in infected fluids. The addition of trichloracetic acid to fractions obtained by ammonium sulfate precipitation produced turbidity equivalent to the protein nitrogen concentration. Ultraviolet absorption curves obtained with the Beckman spectrophotometer disclosed minima of 252 and 290 mμ and maxima of 265 mμ with both dialyzed normal and infected fluids of equal protein concentration (40 mg %). Absorption in this range is characteristic of proteins.

Electrophoretic studies* were made of dialyzed normal and infected allantoic fluids. Normal fluid was concentrated 23-fold, and infected fluid 9-fold, resulting in protein concentrations of 375 and 300 mg %, respectively. These concentrations proved insufficient for accurate analysis of mobilities or sharp delineations of peaks; however, the rate of boundary migration and the degree of boundary spreading were consistent with what would be expected with protein solutions containing several components. With both normal and infected fluids at least 3 peaks were clearly discernible.

Comparison of normal and infected allantoic fluid protein concentrations. Studies of uninfected embryos demonstrated low concentrations of protein in the allantoic fluid as measured by turbidity produced with trichloracetic acid. In embryos of 10-12 days of age turbidity values varied from 8 to 75, representing protein concentrations of 2.4 to 23 mg % with an average value of 8.7 mg %. The frequency distribution of turbidity readings of allantoic fluids from 100 normal embryos is charted in Fig. 2 and compared with the range of turbidity (*i.e.* protein concentration) found in fluids from 41 embryos infected with the Lee strain of influenza virus. Turbidity readings of the infected allantoic fluids ranged from 118 to 540 (*c.f.* Table I), indicating concentrations of 35.4 to 162 mg % of protein.

Lee-infected allantoic fluid was subjected to differential centrifugation to determine to what extent sedimentable substances contributed to turbidity produced with trichloracetic acid. Low speed centrifugation (3,000 r.p.m. for 10 minutes) caused a 12% reduction in the turbidity observed initially, suggesting the presence of considerable cellular material. Total cell counts disclosed an average of 560 cells/cu mm in Lee-infected allantoic fluid contrasted with an average of 93 cells/cu mm in normal fluid. Further centrifugation at 37,900 g for 30 minutes resulted in a further reduction of the turbidity produced with acid amounting to 8% of the turbidity originally present. Thus, Lee virus, itself, contributes little, if any, to the turbidity of infected allantoic fluid, as this amount of centrifugation leaves less than 1% of the virus in the supernate.

Source of protein in infected allantoic fluid. It appeared likely that the increased protein in the allantoic fluid of virus-infected embryos was attributable to host reaction and perhaps to destruction of host tissue, especially in

* Electrophoretic analysis was kindly carried out by Dr. Gertrude E. Perlmann, The Rockefeller Institute, New York City.

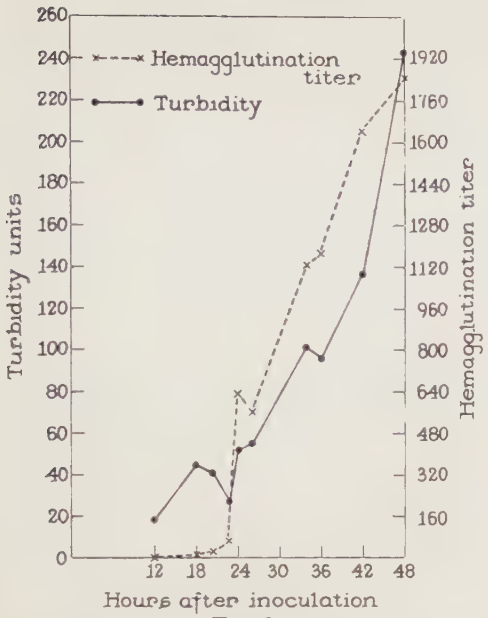

FIG. 3.

Temporal relation between increase in turbidity and Lee virus concentration in allantoic fluid.

view of the increased number of cells in infected fluid. Evidence cited above demonstrated that the virus itself did not contribute to the turbidity produced with acid, and further studies have shown no direct relation between virus and protein concentrations. Moreover, experiments with Lee virus demonstrated a difference in the rate of virus multiplication and the increase in allantoic fluid turbidity as is shown graphically in Fig. 3. Corroboration of this difference was obtained in experiments in which the time required to reach maximal virus concentration was varied by the use of inocula of differing dilutions of virus. The results are presented in Table II.

The protein of infected allantoic fluid did not differ immunologically from the protein normally present in allantoic fluid. Sera from rabbits injected intravenously with dialyzed infected or normal allantoic fluid contained antibodies capable of forming precipitates with concentrated allantoic fluid from either infected or normal embryos. These antibodies were absorbed from either antiserum by normal or infected allantoic fluid antigen, as well as by a suspension of normal chorioallantoic membrane (C.A.M.). These data are summarized in Table III.

Viruses which cause an increase in allantoic fluid protein. The Lee and PR8 strains of influenza virus, Semliki Forest virus, Newcastle disease virus, and mumps virus consistently caused an increase in allantoic fluid protein during the course of infection of the allantoic sac. A correlation may be drawn between the toxicity of the viruses studied and the degree of protein increase. Lee and Newcastle disease viruses, which killed embryos after 48 hours, and Semliki Forest virus, which killed even sooner, caused significantly more turbidity in allantoic fluid than did the considerably less toxic PR8 and mumps strains, as is shown in Table I.

Nonviral causes of protein increase in allantoic fluid. The production of the turbidity reaction by 5 different viruses is evidence of its non-specificity. It is obvious that any reaction dependent upon response or destruction of host tissue may be induced by chemical or physical agents as well as infectious ones. Thus, it was found that injection of 0.1 cc quantities of broth or serum might increase the allantoic fluid protein of embryos beyond the low concentrations usually seen. Such increases were greater than would be anticipated on the basis of the amount of protein injected, demonstrating an actual reaction of the embryonic tissue to the material introduced.

When broth was injected in control embryos, mean turbidity values were almost double (*i.e.* 48 units) those observed in saline inoculated embryos, and occasional fluids exceeded 100 turbidity units. The injection of undiluted allantoic fluid occasioned similar non-specific response, although it was of lesser degree (mean turbidity, 38 units), and only 10% of individual fluids exceeded the upper limit of 75 turbidity units observed in normal embryos (Table I).

The effect of bacterial infection of the chick embryo was not systematically studied, but examination of bacterially contaminated allantoic fluids disclosed increased turbidity, *i.e.* more than 75 units, in only 2 of 16 instances. In any event, such fluids are unsuitable for virus study, and are customarily discarded.

In normal embryos incubated for more

TABLE II.
Relation of Increase in Allantoic Fluid Turbidity to Extent of Multiplication of Lee Virus.

Virus dilution inoculated	Time after inoculation: 24 hr Hem. titer*	24 hr Turb.†	36 hr Hem. titer	36 hr Turb.	48 hr Hem. titer	48 hr Turb.
10^{-3}	1:636	53	1:1331	96	1:1843	243
10^{-5}	0	28	1:1536	93	1:2048	218
10^{-7}	0	21	1:65	42	1:1229	145

* Mean hemagglutination titer of allantoic fluids.
† Mean turbidity value of allantoic fluids.

TABLE III.
Absorption Experiments with Antisera Against Normal and Infected Allantoic Fluid Protein.

Rabbit serum	Absorbed with	Precipitin titer*: Normal all. fl. prot.	Infected all. fl. prot.
Anti normal all. fl. prot.	—	1:250	1:250
" " " " "	Infected all. fl. prot.	0	0
Anti infected all. fl. prot.	—	1:250	1:250
" " " " "	Normal all. fl. prot.	0	0
" " " " "	Normal C.A.M.	0	0

* Highest dilution of antigen which gave a positive reaction with serum diluted 1:2.

TABLE IV.
Prevention of Protein Increase in Allantoic Fluid by Virus Antiserum.

Inoculum: Serum	Inoculum: Lee virus dilution	Mean hemagglutination titer of allantoic fluids	Mean turbidity value of allantoic fluids
—	10^{-6}	1:1877	150
Normal 1:100	"	1:2046	93
" 1:500	"	1:2253	188
Anti Lee 1:100	"	0	25
" " 1:500	"	1:2	19
" " 1:100	—	—	20
" " 1:500	—	—	42

than 12 days, sharp, capricious increases in the allantoic fluid protein may occur, making such embryos unsuitable for use with the present method. Fluids grossly contaminated by blood introduced at the time of harvest also proved useless because of the presence of extraneous serum protein.

Prevention of protein increase by virus antiserum. Neutralization of Lee virus by specific immune rabbit serum prevented the increase in allantoic fluid protein which occurred following the injection of virus and normal serum or virus alone. The results are summarized in Table IV. It will be seen that, in the dilutions indicated, inactivated rabbit serum *per se* caused no undue elevation of mean turbidity. In other experiments dilutions of rabbit serum as low as 1:40 were used without increase in allantoic fluid protein. This experiment affords definitive evidence that the protein increase which follows introduction of virus into the allantoic sac is a corollary of virus multiplication.

Discussion. The study of many animal viruses and attempts to recover new viruses have been handicapped by the lack of simple

in vitro technics comparable to the hemagglutination reaction. The method outlined in the present communication has the virtue of simplicity, and in theory may be of value in the detection of any virus capable of multiplication in the cells of the allantoic sac of the chick embryo. Because the method is dependent upon the vagaries of host response, it is unsuitable for the direct measurement of virus concentration. However, both specificity and quantitation may be obtained by the employment of immune serum. It is conceivable that a virus might be recovered and identified immunologically as the etiological agent of a disease solely by chemical evidence of infection of the chick embryo.

Summary. A method is described for the detection of virus infection of the allantoic sac of the chick embryo. The method is dependent upon the increased concentration of protein in infected allantoic fluid. Protein concentration is measured by determining the degree of turbidity produced upon the addition of 10% trichloracetic acid to allantoic fluid.

While this article was in press, Polson and Dent (*Nature*, 1949, **164**, 233) described increases in the protein concentration of allantoic fluid from eggs infected with lumpy skin disease virus or blue tongue virus.

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17308. Hemagglutination with the GDVII Strain of Mouse Encephalomyelitis Virus.

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The capacity of certain viruses to cause agglutination of erythrocytes[1] has permitted the development of *in vitro* procedures which have greatly facilitated investigative and diagnostic work with these agents. Although at least 10 different animal viruses are known to cause hemagglutination (pertinent data have been summarized recently),[2] there is almost no evidence indicating that any of the neurotropic viruses possesses a similar capacity. Recently, however, Bremer and Mutsaars[3] stated that the Lansing strain of poliomyelitis virus caused agglutination of sheep RBC, and Hallauer[4] stated that Columbia SK and Columbia MM viruses also agglutinated sheep RBC. We have been unable to confirm the results reported for the Lansing strain, as too have other workers.[5] However, in the accompanying paper Olitsky and Yager[5] have confirmed and extended the results reported for SK and MM viruses.

The present study was concerned chiefly with the GDVII strain of mouse encephalomyelitis virus as well as with the FA strain.[6] In addition, the Lansing, MEF1, and Brunhilde strains of poliomyelitis virus were investigated. It will be demonstrated that the GDVII strain causes agglutination of human RBC at 4°C, but not at 23 or 37°C; that such hemagglutination is inhibited by homologous immune serum, and by anti-FA virus serum, but not by antiserum against other viruses. No evidence of hemagglutination could be obtained with the FA strain nor with any of the strains of poliomyelitis virus which were employed.

Materials and methods. Viruses. The

* Aided by a Fellowship from the National Foundation for Infantile Paralysis.

1 Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

2 Smadel, J. E., Viral and Rickettsial Infections of Man, 1948, chap. 3, J. B. Lippincott Co., Philadelphia, Pa.

3 Bremer, A., and Mutsaars, W., *Compt. rend. Soc. Biol.*, 1948, **142**, 1192.

4 Hallauer, C., 4th Internat. Cong. Microbiol., July 20-26, 1947, Copenhagen, 1949, p. 257.

5 Olitsky, P. K., and Yager, R. H., accompanying paper.

6 Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49.

GDVII strain was obtained from Dr. Max Theiler, I.H.D. Laboratories, The Rockefeller Foundation, New York City. Three FA strains were used; one obtained from Dr. Theiler and 2 obtained from Dr. J. Melnick, Yale University, New Haven, Conn. Three poliomyelitis virus strains were employed; the Brunhilde strain was kindly supplied by Dr. D. Bodian, The Johns Hopkins University, Baltimore, Md., and the Lansing and MEF1 strains were obtained from Dr. P. K. Olitsky, The Rockefeller Institute, New York City. The Brunhilde strain was contained in infected monkey spinal cord. The other viruses were maintained by occasional intracerebral passage in mice. Brains were removed from exsanguinated mice shortly after the appearance of signs indicating infection of the central nervous system. Ten per cent brain suspensions were prepared with 0.01 *M* phosphate buffer at pH 7.2. The suspensions were ground for 2½ minutes in a modified Waring Blendor which was cooled with ice and then were centrifuged for 15 minutes at 7,760 g. The supernates were employed either promptly after preparation or following storage at –70°C, sometimes for as long as 14 days. Virus titrations were performed by the intracerebral technic using serial 10-fold dilutions in 10% normal rabbit serum saline. A group of 5 or 6 mice was used for each dilution and the 50% infectivity end point, LD_{50}, was calculated in the usual manner.

Hemagglutination technic. Hemagglutination titrations were carried out in a manner similar to that employed with influenza virus.[1] Serial 2-fold dilutions of brain suspensions in saline buffered at pH 7.2 and a final concentration of 0.25% human Group O erythrocytes were employed. With the GDVII strain the mixtures were held at 4°C for 2 hours. Readings were recorded in the usual manner and the end point was taken as the highest dilution which gave a 2+ reaction.

Hemagglutination-inhibition technic. Antibody titrations were carried out with serial 2-fold dilutions of inactivated (56°C/30 min.) sera in buffered saline and a constant amount of virus, usually 16 hemagglutinating units. A final concentration of 0.25% RBC

TABLE I.

Hemagglutination with GDVII Strain of Mouse Encephalomyelitis Virus.

Supernate of mouse brain suspension	Human group O erythrocytes, %	Held 2 hr at °C	Final dilution of supernate								
			125	250	500	1000	2000	4000	8000	16,000	32,000	64,000
GDVII strain	0.25	4	4*	4	4	4	4	4	3	1	±	0
" "	0.25	23	0	0	0	0	0					
" "	0.25	37	0	0	0	0	0					
" "	2.5	4	4	4	2	0	0	0	0	0	0	0
" "	1.0	4	4	4	4	3	2	1	0	0	0	0
" "	0.5	4	4	4	4	4	3	2	1	±	0	0
" "	0.25	4	4	4	4	4	4	4	3	1	±	0
FA	0.25	4	0	0	0	0	0					
Poliomyelitis, Lansing	0.25	4	0	0	0	0	0					
" MEF1	0.25	4	0	0	0	0	0					
Normal, control	0.25	4	0	0	0	0	0					

* Indicates degree of hemagglutination.

was used and readings were made after 2 hours at 4°C. The end point was taken as the highest dilution of serum which completely inhibited hemagglutination.

Immune sera. Through the courtesy of Dr. P. K. Olitsky immune sera against a large number of different neurotropic viruses were made available. In most instances the sera were obtained from rabbits which had been repeatedly injected intraperitoneally or subcutaneously with infected mouse brain. In some instances sera were also obtained from immunized guinea pigs, mice or monkeys. Immune sera and control normal sera usually were stored at –30°C.

Hemagglutination with GDVII virus. Positive results were obtained in hemagglutination experiments with the GDVII strain when (a) suspensions of infected mouse brain, (b) human Group O erythrocytes, and (c) a reaction temperature of 4°C were employed. The results of typical experiments are shown in Table I. High titers ranging from 1:2,000 to 1:16,000 or more were commonly obtained with 0.25% RBC. In general, the hemagglutination titer was inversely proportional to the concentration of RBC, as is the case also with influenza virus.[7] Hemagglutination occurred only if the mixtures were cold (4°C), disappeared rapidly when cold mixtures were warmed either at room temperature (23°C) or at 37°C, and reappeared when the mixtures were again cooled to 4°C. The reaction developed relatively slowly and, although clear evidence of hemagglutination was present at one hour, more definite agglutination was present at 2 hours. The pattern of agglutinated cells was closely similar to that observed with human RBC and either influenza or mumps virus.

Despite numerous attempts employing a wide range of experimental conditions, it was not possible to obtain evidence of hemagglutination with the FA strain nor with the Lansing, MEF1 or Brunhilde strains of poliomyelitis virus. In addition to human RBC, erythrocytes from the following species were used: monkey, horse, sheep, cat, dog, guinea pig, hamster, mouse and chicken. The GDVII strain was incapable of causing agglutination of any RBC other than those derived from man. Supernates of normal mouse brain suspensions, prepared as described above, did not cause agglutination of human RBC at dilutions greater than 1:4. As is pointed out also in the accompanying paper,[5] erythrocytes of certain species, *e.g.*, hamster, dog, cat and guinea pig, as well as the mouse, commonly showed agglutination when mixed with normal mouse brain suspensions.

The agglutination of human RBC which is caused by GDVII virus in the cold disappears after a few minutes at room temperature. Because of this, both the reaction and readings of titrations are best carried out in the cold room. As is shown below, agglutination is associated with adsorption of the virus to RBC and the dispersal of the agglutinated cells is associated with elution of the virus from them. Successive cycles of adsorption and elution, dependent merely on changes in temperature, can be repeated at will with a single mixture of RBC and GDVII virus. Four such cycles have been carried out.

Hemagglutination-inhibition with immune serum. Agglutination of human RBC with GDVII virus in the cold was prevented by high dilutions of anti-GDVII serum as well as anti-FA serum but not by immune serum against other viruses. The results of typical experiments are shown in Table II. Hemagglutination-inhibition titers ranging from 1:4,000 to 1:16,000 were obtained commonly with anti-GDVII serum and similar high titers were obtained also with anti-FA serum. Normal serum usually caused some non-specific inhibition; with rabbit and guinea pig serum titers of 1:32 or lower were commonly found; with human and mouse serum titers as high as 1:128 were encountered. In some instances heating at 56°C for 30 minutes reduced the degree of non-specific inhibition. It should be emphasized that the injection of mouse brain suspensions into animals other than mice commonly results in the development of antibodies which cause agglutination of human RBC. Usually the agglutination titer of such sera is not greater than 1:128 but in occasional instances it may be considerably higher. With immune serum agglutination of RBC occurs not only at 4°C but also

[7] Whitman, L., *J. Immunol.*, 1947, **56**, 167.

TABLE II.
Inhibition of Hemagglutination with GDVII Virus by Immune Serum.

Serum		GDVII virus units	Held 2 hr at °C	Serum hemagglut. inhibition titer
Immune vs.	Species			
Normal m.br*	Rabbit	16	4	0†
GDVII m.br.	"	16	4	8000
Normal m.br.	Mouse	16	4	0
FA m.br.	"	16	4	8000
" "	Monkey	16	4	8000
Poliomyelitis (conval.)	"	16	4	0
" Lansing m.br.	Rabbit	16	4	0
Mengo, m.br.	"	16	4	0

* m.br. = Mouse brain.
† O = No inhibition of hemagglutination at 1:32 serum dilution.

at room temperature or at 37°C, as not with GDVII virus. The agglutinins were readily removed from such sera by absorption with 20% human RBC at 4°C. Absorbed immune sera gave hemagglutination-inhibition titers with GDVII virus which were identical with those obtained with unabsorbed sera.

Immune sera against the following viruses were employed in hemagglutination-inhibition experiments with GDVII virus: lymphocytic choriomeningitis, Eastern equine encephalitis, Japanese B encephalitis, St. Louis encephalitis, Russian Far East encephalitis, vesicular stomatitis, West Nile, rabies, herpes simplex, vaccinia, Columbia SK, Columbia MM, Mengo encephalomyelitis, encephalomyocarditis, influenza A (PR8 strain) and PVM. In no instance was significant inhibition of GDVII virus demonstrable with these sera.

In cross-immunity experiments Theiler and Gard[6] demonstrated an immunological relationship between GDVII and FA viruses. It appears of considerable interest that the results of hemagglutination-inhibition experiments indicate not only that antibody specifically directed against GDVII virus is present in high titer in immune sera but also show clear evidence of a close antigenic relationship to FA virus.

Adsorption and elution of GDVII virus. When mixtures of human RBC and GDVII virus were held at 4°C, the virus was adsorbed rapidly by the erythrocytes and sedimented with them on light centrifugation. When the sedimented RBC were resuspended in buffered saline and held at 4°C, elution of the virus did not occur. However, when the resuspended RBC were warmed to 37°C, elution of the virus occurred very rapidly and maximum titers were obtained in the supernate within 5 to 10 minutes. Results of typical experiments are shown in Fig. 1. As would be expected, the concentrations of RBC employed affected the extent to which the virus was adsorbed at 4°C but did not have any striking effect on the rate or degree of elution at 37°C. Mixtures held at 4°C for as long as 24 hours showed no significant elution of the virus from RBC.

That hemagglutination with GDVII virus is caused by the virus particle itself and not by a component separable from the virus is

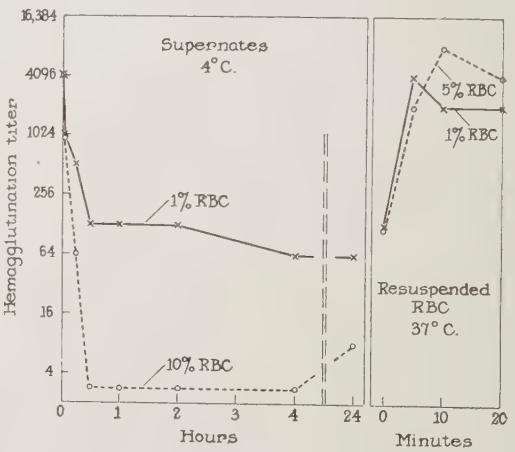

FIG. 1.

Adsorption of GDVII virus on human Group O RBC at 4°C and elution at 37°C. Hemagglutination titer of supernates from mixtures of virus and RBC is plotted against time mixtures were held at 4°C. Hemagglutination titer of supernates from resuspended RBC is plotted against time such erythrocytes were held at 37°C.

TABLE III.
Adsorption on and Elution from Human RBC of GDVII Virus.

Material tested	Hemagglutination titer* at 4°C vs. human RBC	Virus infectivity titer* in mice I.C., LD_{50}
GDVII m.br. suspension	10,240	$10^{-8.1}$
Supernate after adsorption with 10% RBC, 1 hr at 4°C	160	$10^{-5.6}$
Supernate of resuspended RBC in saline, 15 min. at 37°C	10,240	$10^{-7.8}$

* Titers are expressed in terms of final dilution of brain material.

indicated by the results shown in Table III.

Adsorption of a suspension with human RBC at 4°C resulted in reductions in the hemagglutination and virus infectivity titers of the supernate which were of similar degree. Moreover, on warming the resuspended RBC at 37°C, similar increases in both titers occurred indicating that elution of the virus was effected at the higher temperature.

Properties of hemagglutination component. The hemagglutination titer of GDVII mouse brain suspension was not diminished by storage at 4°C for 43 days. Heating crude suspensions in saline at 56°C for 30 minutes caused marked loss, *i.e.* 99%, of hemagglutinating capacity. On the other hand, suspensions prepared from brain material extracted by methanol in the cold showed only a 2- to 4-fold reduction in titer on similar heating. Moreover, such suspensions showed hemagglutination titers of 1:1,000 after heating at 65°C for 30 minutes. Centrifugation at 7,760 g for 30 minutes did not reduce the hemagglutination titer of suspensions. Filtration through Seitz-EK pads caused an 8-fold reduction in the titer of the filtrate. Suspensions buffered at pH values from 4.8 to 8.3 gave similar titers. The amount of virus adsorbed by human RBC at 4°C was not significantly affected by the pH of the mixture within this range. Furthermore, elution of virus from RBC at 37°C was complete when erythrocytes were resuspended in buffer of pH 4.8 to 8.

Concentration of GDVII virus. By resuspension of RBC with adsorbed virus in small volumes of buffered saline and warming the suspension to 37°C, it was possible to achieve considerable concentration (10 times or more) of the virus in the eluate. In most instances the increase in titer obtained was as great as or greater than would have been expected in terms of the volumes of eluate employed. Either high or low titer suspensions as well as suspensions which had been diluted before adsorption yielded satisfactory results in concentration experiments of this kind.

Recently it was reported[8] that GDVII virus could be purified considerably by precipitation with 25 to 30% methanol in the cold. In the present study numerous attempts were made to concentrate the virus by means of such a procedure. In all instances the virus titer was determined by the hemagglutination technic. It was found that 52% methanol mixtures held at 4°C for 3 hours yielded better results than mixtures at other methanol concentrations. Despite the use of a large variety of experimental conditions which included variations in pH, ionic strength, amount of centrifugation before and after the addition of methanol, as well as extraction of brain material with organic solvents, it was not possible to obtain consistent results. In some experiments 10-fold or greater concentration was achieved but the results were not sufficiently reproducible to make the procedure valuable. Moreover, after concentration by methanol precipitation, the virus appeared to be unstable and hemagglutination titers decreased rapidly on storage of concentrated material at 4°C.

Failure of FA virus to cause hemagglutination. The infectivity titer of GDVII virus is

[8] Brumfield, H. P., Stulberg, C. S., and Halvorson, H. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 410.

definitely higher than that of FA virus. With the strains employed in this study, GDVII gave LD_{50} titers of the order of 10^{-8} or more while FA gave titers of the order of 10^{-6} or less. On the assumption that the infectivity titer is proportional to the virus concentration, it seemed possible that the failure to demonstrate hemagglutination with FA might be attributable to a relatively low concentration of the agent in infected brain tissue. Because of the numerous similar properties of the 2 viruses[6] and the close antigenic relationship disclosed in the hemagglutination-inhibition experiments described above, it appeared desirable to determine if FA shared with GDVII the capacity to cause hemagglutination of human RBC in the cold.

Attempts to concentrate FA virus, as was feasible with GDVII, by adsorption on human RBC at 4°C and elution in a small volume of diluent at 37°C, were uniformly unsucessful. In no instance was hemagglutination demonstrable with the eluates despite the use of erythrocytes derived from numerous species. Moreover, the results of infectivity titrations indicated that FA virus was not adsorbed by human RBC under the conditions employed; supernates of virus-RBC mixtures held at 4°C showed no reduction in infectivity titer. It appears, therefore, that despite similarities relative to numerous properties FA virus and GDVII virus do not give similar reactions with human erythrocytes *in vitro*.

Discussion. That certain neurotropic viruses possess the capacity to agglutinate in the cold erythrocytes deriving from certain animal species appears evident from the results of this study and that described in the accompanying paper.[5] By means of the hemagglutination reaction with human RBC at 4°C it is possible to estimate *in vitro* the concentration of GDVII virus in a suspension of infected mouse brain. As is the case with other animal viruses which cause hemagglutination, relatively high concentrations are required before positive results are obtained; with GDVII of the order of 10^4 mouse infectious doses of virus correspond to one hemagglutinating unit. The available evidence suggests that the infective virus particle is itself responsible for hemagglutination with this agent. By means of the hemagglutination-inhibition technic, also carried out with human RBC at 4°C, the concentration of antibodies in immune serum specifically directed against the virus can be estimated *in vitro*. Evidence obtained in hemagglutination-inhibition experiments indicates that GDVII virus is immunologically closely related to FA virus, but is not related to any other of the numerous agents tested.

Despite numerous attempts with a wide variety of experimental conditions, it was not possible to demonstrate hemagglutination with FA virus. Moreover, with the Lansing, MEF1 and Brunhilde strains of poliomyelitis virus no evidence was obtained indicative of a capacity to combine with erythrocytes. It may be pertinent that neither FA virus nor poliomyelitis virus reaches high titers in infected central nervous system tissue and it is possible that the failure to show hemagglutination with these agents is attributable to insufficient concentration. On the other hand, qualitative factors also may be of critical importance and it seems possible that with erythrocytes from still other species and with different experimental conditions positive results might be obtained.

The dependence of hemagglutination with GDVII virus and of adsorption of the agent by human RBC upon a low temperature, *i.e.* 4°C, appears to be unique. With the exception of the neurotropic viruses discussed in the accompanying paper,[5] other viruses which cause hemagglutination show no such temperature effect.

Summary. Suspensions of mouse brain infected with the GDVII strain of mouse encephalomyelitis virus cause agglutination of human Group O RBC at 4°C. Anti-GDVII virus serum inhibits hemagglutination by the agent as also does anti-FA virus serum. GDVII virus is adsorbed by human RBC at 4°C and rapidly elutes from them at 37°C. Three strains of poliomyelitis virus failed to show any evidence of hemagglutination.

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17309. Hemagglutination by Columbia SK, Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses: Experiments with Other Viruses.

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During the course of a study on hemagglutination by certain neurotropic viruses, a study suggested by the findings reported in the foregoing paper,[1] the writers' attention was directed to two reports. One[2] stated that the Lansing strain of poliomyelitis virus, and the other[3] that the Columbia SK (Col SK) and Columbia MM (Col MM) viruses agglutinated sheep erythrocytes, the agglutination being inhibited by specific antisera.

In our own work no hemagglutination by the Lansing strain was found; Hallauer[3] also reported failure. On the other hand, hemagglutination of sheep RBC by Col SK and Col MM viruses[3] was not only confirmed in the present investigation but a similar specific reaction was also obtained with Mengo encephalomyelitis (ME) and encephalomyocarditis (EMC) viruses.

This paper reports the results of these tests as well as attempts to disclose agglutination of sheep red cells and several additional types of erythrocytes by still other neurotropic viruses. Furthermore, there will be described an agglutinin for erythrocytes deriving from several species of animals present in suspensions of normal mouse brain, as well as an inhibitor of agglutination contained in the tissue suspensions and also in normal serum.

Hemagglutination of Sheep Erythrocytes by Col SK, Col MM, ME, and EMC Viruses. Dick and Taylor[4] have employed solutions of crystalline bovine plasma albumin (BPA) as a medium for preservation and for dilution of several viruses, among which were influenza, yellow fever, Lansing and ME. A 0.1 or 0.2% solution of the crystals in buffered saline solution, filtered through a Seitz apparatus generally sufficed for ordinary laboratory purposes, especially for dilution in titration tests.[4] It was also found in this laboratory that for the several viruses employed in the present study BPA was a satisfactory vehicle and the advantages of utilizing a clear, nonagglutinating solution, which also preserved the titer of a virus in tests for hemagglutination, were apparent. Consequently virus-infected mouse brains, (20%) were suspended preferably in 0.1% BPA although saline solution or 10% rabbit serum could also be employed. Such suspensions were used in the fresh state; or if stored, were kept frozen in a mechanical, electrically operated freezer at -20 to -25°C and thawed just before use. Dilutions of virus for hemagglutination were made, however, with buffered saline solution, 0.85% NaCl, 0.05 M phosphate and pH 7.6.

The procedure of the test was as follows: The fresh or thawed viral suspension consisting of 20% brain tissue was centrifuged for clarification at 2,000 rpm for 5 minutes. 0.4 ml of the supernate was added to the first 2 of a series of 10 to 15 tubes. Buffered saline solution in equal amount was introduced into all tubes except the first to make a 2-fold dilution in a final volume of 0.4 ml in the second and successive tubes. To each virus dilution was added 0.4 ml of 0.5% washed sheep RBC suspended in buffered saline solution, thus securing final dilutions of virus of 1:10 to 1:5, 120, or higher, and of the erythrocytes in each tube, 0.25%. Cells were prepared from fresh bleedings and stored in modified Alsever's fluid (ACD);[4a] as such they could be kept for about 1 month in the ice

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1 Lahelle, O., and Horsfall, F. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 713.

2 Bremer, A., and Mutsaars, W., *C. r. Soc. biol.*, 1948, **142**, 1194.

3 Hallauer, C., 4th Internat. Cong. Microbiol., July 20-26, 1947, Copenhagen, 1949, p. 257.

4 Dick, G. W. A., and Taylor, R. M., *J. Immunol.*, 1949, **62**, 311.

4a Rapoport, S., *J. Clin. Invest.*, 1947, **26**, 591.

TABLE I.
Hemagglutination of Sheep Cells by Columbia SK, Columbia MM, Meningo Encephalomyelitis and Encephalomyocarditis Viruses, Held for 120 Min. at 5°C.

	Virus or control materials	Reciprocal of final dilution of virus or of normal mouse brain							RBC of types other than sheep, plus virus (10 to 5,120 dils.)
		10	20	40	80	160	320	640 to 5,120	
Test	Col SK	4	4	2	±	0	0	0	0
	Col MM	4	4	2	0	0	0	0	0
	ME	4	3	3	3	2	±	0	0
	EMC	4	4	4	3	2	1	0	0
	West equine	0	0	0	0	0	0	0	0
	East "	0	0	0	0	0	0	0	0
	GDVII	0	0	0	0	0	0	0	+ with human-o cells only
Controls	Normal mouse brain	0	0	0	0	0	0	0	See text
		sheep cells							Cells alone
	Saline soln.	0							0
	BPA	0							0

box; after washing, however, not longer than 5 days. Hamster erythrocytes were prepared from fresh bleedings and were not satisfactorily stored; they were proved useless if kept in ACD for periods longer than 4 days. The tubes were shaken, kept for 60-120 minutes at 5°C and then read. It should be emphasized here that false positives, *i.e.*, nonspecific reactions, easily obscured the results especially since mouse-brain suspensions were employed; the cause of this difficulty will soon be given. Hence tests should include a) a control on the virus suspension, namely, normal mouse brain suspension and b) antiserum to determine the specificity of hemagglutination. Generally the methods here described and the scale of reading agglutination, except for certain modifications, follow those already described.[1,5] It should be stressed here also that even slight variations in technic sometimes brought about irregular results —a fact which applies also to the standard test.[5]

Table I shows the outcome of one of several similar experiments. The selective agglutination of sheep RBC by Col SK, Col MM, ME and EMC viruses is noted. It is not surprising to find such uniformity of reaction exhibited by the 4 viruses since it has already been found by Warren and Smadel[6] and by Dick[7] that there is a close relationship among the members of this group as proved by the results of serological, immunological and other biological studies.

With these 4 viruses the hemagglutination was carried out best at 5°C for the reason that spontaneous elution or dispersion of virus from the erythrocytes occurred at room (23°C) or incubator (37°C) temperatures so that at the higher temperature little or no agglutination was visible. This reversibility of the agglutination by means of increasing the temperature could be carried out with the same materials for an indefinite number of times, or as long as sufficient virus survived the process to show its hemagglutinative capacity. In this respect, the present group of viruses behaved as did the GDVII virus.[1]

Specificity of Hemagglutination. The next investigation related to the specificity of the hemagglutination for sheep erythrocytes by Col SK, Col MM, ME, and EMC viruses. The procedure followed the established principles[5] of a preliminary titration to determine the hemagglutination titer of the virus to be tested and of selection of a dilution of it which represented not less than 4 and not more than 8 agglutination units. 0.2 ml of this dilution of virus was added to 0.2 ml of antisera, then 0.4 cc of the cells—no preliminary

[5] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49; Smadel, J. E., in Viral and Rickettsial Infections of Man, ed. T. M. Rivers, J. B. Lippincott Co., Philadelphia, 1948, Chap. 3, pp. 77-82.

[6] Warren, J., and Smadel, J. E., *J. Immunol.*, in press.

[7] Dick, G. W. A., *J. Immunol.*, in press.

TABLE II.
Hemagglutination-Inhibition by Antisera: Col SK, Col MM, ME and EMC Viruses and Sheep Cells.

Virus	Antiserum*	Virus. agg. units	Reciprocal of final dilution of serum							
			40	80	160	320	640	1,280	2,560	5,120
Col SK	Col SK	4	0	0	0	0	0	0	0	4
Col SK	NRS†	4	4	4	4	4	4	4	4	4
Col MM	Col MM	4	0	0	0	0	0	1	2	3‡
Col MM	NRS	4	4	4	4	4	4	4	4	4
ME	ME	8	1	0	0	0	0	0	3	4
ME	NRS	8	4	4	4	4	4	4	4	4
EMC	EMC	8	1	0	0	0	0	2	4	4
EMC	NRS	8	4	4	4	4	4	4	4	4
ME	WEE§	8	4	4	4	4	4	4	4	4
EMC	WEE	8	4	4	4	4	4	4	4	4

* All antisera were prepared by injecting rabbits repeatedly with mouse-brain virus.
† NRS = normal rabbit serum.
‡ The titration continued as follows: 10,240 read 3; 20,480 read 4.
§ Western equine encephalitis antiserum.

period of incubation of antisera and virus was found necessary. The antisera used were prepared by injecting rabbits subcutaneously 3 times at weekly intervals with 1, 2, and 3 respectively, fresh or frozen virus-infected mouse brains. All serum whether immune or normal was inactivated by heating at 56°C for 30 minutes. The virus was kept constant at 4 or 8 units per tube and the serum was diluted 2-fold beginning with 1:10, thus the final dilutions of serum became 1:40 to 1:20,480, since 10 dilutions were usually tested. The test was read after 60 minutes at 5°C. The reading of the hemagglutination inhibition followed standard methods.[5]

Table II demonstrates one of the tests. It will be observed that not only did the antisera inhibit specifically the hemagglutination of sheep cells by the Col SK group of viruses but there was evidence of clear-cut cross-reactions among members of this group of viruses. Table III is presented to demonstrate the results of a test on inhibition of hemagglutination, the titer of antisera and certain cross-reactions among the members of the Col SK group of viruses.

TABLE III.
Tests Showing Hemagglutination-Inhibition Titers of Antisera Against Col SK Group of Viruses and Certain of the Cross-Reactions.

Virus	Antiserum	Agglutination-Inhibition titer
Col SK	Col SK	1:2,560*
	Col MM	1:640
	ME	1:1.280
	EMC	1:1,280
ME	ME	1:2,560
	EMC	1:320
EMC	EMC	1:640
	Col SK	1:640

* The dilutions represent the highest dilution of antiserum preventing agglutination (Table II).

It is concluded therefore that the Col SK, Col MM, ME and EMC viruses agglutinate sheep RBC specifically; they exhibit cross-agglutination inhibition among the individuals of the group and since other neurotropic viruses do not agglutinate sheep cells, as will be shown immediately, these 4 infective agents can be looked upon as having a generic relationship and a common antigenicity. Thus support is given to the findings of Warren and Smadel[6] and of Dick[7] who produced solid evidence from a wholly different approach to the problem of interrelationship of the 4 agents.

Since the hemagglutination is characteristic, it can apparently be utilized for the identification of the viruses of the Col SK group and for measurement of the antibody content of antisera against any of the 4 agents. Finally, since all of the individual members of the group agglutinate sheep RBC in the cold, elute or disperse spontaneously and rapidly at moderate elevations of temperature, and when

freed from the erythrocytes are as active as they were originally, hemagglutination may be useful for purposes of adsorption, without inactivation, of the active agents, just as can be done with the GDVII virus.[1]

Agglutination Tests with Other Neurotropic Viruses and a Variety of RBC. A wide variety of erythrocytes other than those of sheep, namely, human 0, chick, horse, hamster, dog, cat and guinea pig, were tested for agglutination by the 4 viruses of the Col SK group; the tests failed. In addition, the various erythrocytes just mentioned, including sheep cells, were tested for agglutinability by numerous neurotropic viruses. The viruses employed were:

Eastern equine encephalitis	Theiler (FA strain)
Western equine encephalitis	Theiler (TO strain)
Venezuelan equine encephalitis	poliomyelitis (Lansing strain)
Japanese B encephalitis	poliomyelitis (MEF1 strain)
St. Louis encephalitis	
Russian Far East encephalitis	West Nile
vesicular stomatitis (New Jersey strain)	rabies
vesicular stomatitis (Indiana strain)	lymphocytic choriomeningitis
	herpes simplex
	louping ill

The results of over 100 experiments can be summarized by stating that no specific clumping of any of the types of erythrocytes by any one of the viruses mentioned was detectable. Now and again agglutination was seen but further study revealed it to be nonspecific, most often owing to the physical or particulate condition of the mouse-brain suspension. It was shown that a) normal mouse-brain suspensions produced similar hemagglutination; b) centrifugation to a degree which clarified the suspension but did not sediment the virus from the supernate served to abolish the agglutinative capacity of the supernate, c) antisera failed to inhibit the reaction, d) the reaction was not reversible, e) the aggregations formed did not resemble the "soft", clinging, fine agglomerations as was seen in the hemagglutination by Col SK group of viruses just described. On the contrary, the aggregates were usually "hard", coarse, irregularly sized and shaped, and sometimes were surrounded by a narrow zone of slight hemolysis.

In view of the fact that GDVII virus exhibits agglutination only of human-O RBC[1] and the Col SK group of viruses only of sheep cells, one may well question the meaning of the negative results obtained with the other neurotropic viruses and the kinds of erythrocytes used in the present investigation. Since agglutination is so selective with respect to cells used, it will not be surprising to find one or another of these viruses yielding positive results with erythrocytes deriving from species not as yet tested.

Agglutinative Capacity of Normal Mouse Brain Suspensions for Dog, Cat and Guinea Pig RBC. During the course of the present study, it was noted that suspensions of normal mouse brain agglutinated to a low degree, dog, cat, and guinea pig RBC. The hemagglutination titers were generally 1:10, less often 1:20, rarely 1:40, and of 15 samples in one instance only, 1:320. This reaction was observed after 60 minutes at 5°C, but the maximum titer was reached, however, at room temperature. The reaction was not reversible: there was no visible phenomenon similar to that of spontaneous elution. Moreover, antisera prepared by immunizing rabbits against normal mouse brain had no inhibitory effect on the agglutinative power of the normal mouse brain. The agglutination thus produced revealed, therefore, characteristics unlike those of the viruses of the Col SK group. It is clear, however, that since neurotropic viruses are often employed in the form of infected mouse brain, the occurrence of this nonspecific hemagglutination should be reckoned with in experimental studies.

Presence of an Agglutinin and an Agglutination-Inhibitor for Hamster Cells in Normal Mouse Brain. It was also disclosed during the course of the present investigation that there exists in normal mouse brain, and therefore in virus suspensions prepared with infected mouse brain, an agglutinin as well as an agglutination-inhibitor (HI) for hamster erythrocytes, both being present in the same suspensions at the same time.

TABLE IV.

Presence of Hemagglutination-Inhibitor and Agglutination of Hamster RBC by Normal Mouse Brain (NMB) and Virus-Infected Mouse Brain (120 min. at 5°C).

Material used	Dilution of brain tissue or of antiserum*											
	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960	81,920
NMB	0	0	0	0	0	3	4	4	4	4	4	4
NMB + its antiserum	0	0	0	0	0	0	2	2	3	3	—	—
NMB + NRS*	0	0	0	0	±	1	4	4	4	4	—	—
West equine virus	0	0	0	1	3	4	4	4	4	4	4	2
West equine virus + its antiserum	0	0	0	1	2	4	4	4	4	—	—	—
NMB heated, 56°C, 10 min.	4	4	4	4	4	4	4	4	4	4	4	4
NMB heated, 56°C, 30 min.	4	4	4	4	4	4	4	4	4	4	2	0

* NRS = Normal rabbit serum; in an HI test, 8 units of normal or virus-infected mouse brain was used in each tube; reciprocal of final dilution is given.

The agglutinin for hamster cells present in normal and virus-infected mouse brain became evident at ice box or room temperature, maximal after 2 hours' incubation. The agglutinated masses of hamster cells which formed were more minute, delicate and evenly dispersed along the sides of the test tube than were the aggregates formed by the Col SK group of viruses. Specific antisera did not inhibit the hamster-erythrocyte agglutination to any greater extent than did normal serum of the same species. As will be shown later all sera contained a nonspecific inhibitor. It is therefore plain that this agglutinin has characteristics which differ from those shown by the Col SK group of viruses in the presence of sheep cells. The hamster-RBC type agglutination also differs from that observed when normal or infected mouse brain reacted with dog, cat or guinea pig erythrocytes.

The HI exerted its influence in dilutions of 1:40 to 1:160, rarely (3 of 18 titrations) as high as 1:640. After the HI was diluted out hemagglutination was then visible and showed itself in dilutions usually up to 1:80,000 and sometimes higher (Table IV). The HI was active both at ice-box and room temperatures and the maximum titers were noted after 2 hours' incubation. The inhibitor was thermolabile and could be inactivated by heating mouse-brain suspensions at 56°C for 10 to 30 minutes at which temperature the agglutinin was not affected (Table IV). Sodium citrate 2.5% did not neutralize its effect as it does in the case of PVM and other viruses.[8] It could not be sedimented out of a suspension of mouse brain at 3,000 rpm for 10 minutes and rabbit antisera prepared by repeated injection of normal or virus-infected mouse brain failed to reduce its titer of activity.

Nonspecific HI in Certain Normal Serum and Antisera. Antisera prepared in rabbits, guinea pigs, or monkeys, and the respective normal sera of these species possessed the capacity to inhibit hamster-cell agglutination by normal or virus-infected mouse-brain suspensions. Thus the nonspecific inhibition could be demonstrated in dilutions of sera up to 1:1,280 (Table IV). When normal guinea pig or rabbit serum was heated at 65°C for 30 minutes the contained nonspecific inhibitor was not thereby inactivated. It has been previously reported[8,9] that such treatment of serum can inactivate the inherent nonspecific inhibitor of hemagglutination by other viruses.

[8] Ginsberg, H. S., personal communication.

[9] Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, **89**, 37.

Summary of Nonspecific Agglutinins and Inhibitors. In sharp contrast to the clearly defined, specific agglutination of sheep red cells by the Col SK group of viruses on one side, and the failure of many other neurotropic viruses to show hemagglutination on the other, is the existence of the following nonspecific elements:

a) An agglutinin for dog, cat and guinea pig erythrocytes is contained in normal, or virus-infected mouse brain.

b) An agglutinin for hamster red cells is present in normal or virus-infected mouse brain.

c) A thermolabile inhibitor of agglutination of hamster RBC exists concomitantly with the agglutinin for the hamster cells just mentioned, in normal or virus-infected mouse brain.

d) A thermostable inhibitor is present in normal monkey, rabbit and guinea pig serum, consequently in antisera as well, which prevents agglutination of hamster cells.

The nonspecific reactions just described require further study for the identification of the agglutinins and the inhibitors present in mouse brain suspensions and in normal serum. For the moment, the use of erythrocytes deriving from hamsters, dogs, cats and guinea pigs for agglutination by neurotropic viruses in the form of mouse-brain suspensions would appear to require caution. Since all the standard hemagglutination tests, now routine in laboratory practice, require careful control of the variable employed, similar precautions are needed as well for the Col SK, Col MM, ME, and EMC viruses.

Conclusions. Evidence has been brought forward to indicate that Columbia SK, Columbia MM, Mengo encephalomyelitis and encephalomyocarditis viruses agglutinate sheep red cells. It is therefore possible to identify these viruses by means of hemagglutination and to measure the antibody content of antisera. Since the viruses show characteristic spontaneous elution or dispersion from the erythrocytes, the method can be used for purposes of selective adsorption of the viruses without loss of their hemagglutinative activity. Moreover, the uniformity of the hemagglutination reaction shown by the 4 viruses and the cross-inhibition that exists among them supports the findings of Warren and Smadel[6] and of Dick[7] that these viruses are similar in many repects and are of the same group.

Seventeen other neurotropic viruses were tested for their capacity to agglutinate characteristically erythrocytes deriving from sheep, man (group 0), chicken, horse, hamster, dog, cat and guinea pig; these tests failed.

Another phenomenon that was observed is the nonspecific agglutination of dog, cat and guinea pig erythrocytes by normal or virus-infected mouse brains. With respect to agglutination of hamster cells, an inhibitor of agglutination is present not only in suspensions of normal mouse brain but also in normal serum and antiserum against the neurotropic viruses.

The fact that neurotropic viruses are often used in the form of mouse brain suspensions renders it important therefore for investigators to use proper controls for the variable of the test and, in addition, to identify positive reactions by specific means.

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17310. Veriloid, a New Hypotensive Extract of Veratrum Viride.*

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The promising results of Freis *et al.*[1,2] in therapy of patients with essential hypertension suggested that a hypotensive extract of constant composition from *Veratrum viride* would be highly useful. Accordingly a series of fractionations was carried out.‡ An assay routine based on hypotensive properties in normal dogs under pentobarbital anesthesia was applied. Accessory screening technics included emetic and bradycardic potency estimations. After screening some 75 fractions, a highly potent, reproducible, stable and uniform extract was selected for further study. This material has been given the proprietary name "Veriloid."

While the physical and chemical properties of "Veriloid" will be treated *in extenso* elsewhere a brief outline is presented in Table I. Evidence exists that "Veriloid" is a mitxure and not a single alkaloid. None of the previously described potent alkaloids of *Veratrum viride* has been obtained by fractionation of "Veriloid." (See Krayer[3] for comprehensive review of literature). The relatively impotent rubijervine and isorubijervine have been identified as present in the mixture to the extent of 25%. For clinical use further purification at present was not warranted for logistic reasons. A series of 12 separate fractionations from crude root has shown inappreciable variations in product by both chemical and biological tests.

Biological effects of "Veriloid" are outlined in Table II. Full data will be published separately.

The data illustrate a high therapeutic ratio for hypotensive action. With increasing intravenous dosage in normotensive animals, the degree of hypotension increased only up to an average maximum fall of 40% of the pre-existing mean arterial pressure. As dosage was raised the duration of the reduced mean pressure increased. Usual duration at fully effective intravenous dosage was 30 to 90 minutes. The return to previous pressure was gradual. Preliminary data indicated that the role of the bradycardia in the hypotension was a secondary one. Hypotension still was elicited after vagotomy; bradycardia was not. The hemodynamic rearrangement seen during this hypotension would best fit a hypothesis that "Veriloid" caused dilatation of arterioles in skeletal muscle, splanchnic region and skin accompanied by constriction of venous vascular beds. Evidence was elicited that blood flow was not reduced with the hypotension. Systolic and diastolic blood pressures were both decreased and nearly equally so. Anesthesia did not alter the minimal hypotensive dose. Hypotension resulting from "Veriloid" was corrected by pressor amines such as epinephrine, phenylephrine and methoxamine.

Minimal bradycardic action from intravenous administration had a higher minimal effective dose, a longer latency and a shorter duration than did the hypotensive action. Pentobarbital anesthesia reduced the amount of drug required to produce bradycardia. As the dose was raised degree and duration of bradycardia increased. Lengthening of P-R interval, partial heart block, and transient A-V nodal rhythm were seen at 20 to 50 times the minimal dose. As the dose was raised still further ventricular tachycardia, venous and arterial hypertension resulted. A coarse ventricular fibrillation was observed occasionally at 200 to 500 times the minimal dose. Very high intravenous dosage in the unanesthetized

* Supported in part by a grant from the Coe Chemical Co., Los Angeles, Calif.

† Product Development Department, Coe Chemical Co.

1 Fries, E. D., and Stanton, J. R., *Am. Heart J.*, 1948, **36**, 723.

2 Fries, E. D., Stanton, J. R., Culbertson, J. W., Litter, J., Halperin, M. H., and Wilkins, R. W., *J. Clin. Inv.*, 1949, **28**, 353.

‡ In the laboratories at Los Angeles.

3 Krayer, O., and Acheson, G. H., *Physiol. Rev.*, 1946, **26**, 383.

TABLE I.
Physical and Chemical Properties of "Veriloid."

Appearance: Pale yellow amorphous powder.

Melting Point: Sinters at 102°-105°C.
Melts at 148°-155°C.

Optical Rotation: a $\overset{26^\circ}{D}$ in EtOH = —17.3°C = 0.5 g/100 ml.

Ultra Violet Absorption Curve: Peak at 2500 Å ln E = 3.30 (at 0.00008 g/ml).

Solubility: In water—very slightly soluble.
In dilute acid—soluble.
In benzene, alcohol, chloroform, propylene glycol, acetone—soluble.

Spot test reaction with c. H_2SO_4—Dark orange going to reddish orange to brown in 24 hours.

Nitrogen Content—2.9%.

TABLE II.
Dosage of "Veriloid" in mg/kg Required to Produce Physiological Alterations in Dogs.

Effects	Anesthetized (Pentobarbital sodium) Intravenous	Unanesthetized	
		Intravenous	Oral
Hypotension (reduction of at least 10 mm Hg)	ED50 0.0021 ± 0.0001* (200 trials)	ED50 *ca* 0.002 (26 trials)	Irregular
Bradycardia	ED50 0.0021 ± 0.0003 (75 trials)	ED50 0.0055 ± 0.0004 (75 trials)	Not obtained at 0.05 (20 trials)
Emesis—Fasted	Varies with depth of depression. Rare		ED50 0.0136 ± 0.0017 (50 trials)
Not fasted		ED50 0.0197 ± 0.001 (50 trials)	ED50 *ca* 0.025 (30 trials)
Apnea (30 to 90 sec. duration)	Irregular but never below 0.02 (200 trials)	ED50 *ca* 0.1 (15 trials)	Never obtained (Tried to 5.0)
Hyperirritability (15 to 20 sec. duration)	Never obtained	Above 0.01 (45 trials)	Never obtained
Displacement of cardiac pacemaker	Approximately 0.1 (20 trials)	Never below 0.05. Occasionally at 0.1. Common at 0.2 to 0.5	Seen at 1.0
Hypertension	ED50 *ca* 0.05 (30 trials)		
Lethal	Varies 0.05 to 0.4 (30 trials)	LD50 *ca* 0.5 (6 trials)	Never obtained 5.0 tolerated (5 trials)

* Standard error (See Miller and Tainter, PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 261).

animal produced similar effects except that fibrillation has never been observed. Transient A-V nodal rhythm has also been obtained after oral administration of 1 mg/kg in the dog.

Tachyphylaxis to the bradycardic and hypotensive actions has not been seen. Similarly, tolerance did not develop in 2 dogs following the intramuscular administration of 5 times the minimal effective dose twice a day

for 5 weeks.

Like other Veratrum derivatives "Veriloid" is a potent emetic by any route. Oral dosage required to produce vomiting was at least doubled by the presence of food in the stomach. Thus reduction of clinical side effects would be expected to result from administration during meals.

Oral administration in dosage up to 0.075 mg/kg to the unanesthetized dog has only irregularly resulted in hypotension. The same dose did cause a fall of mean arterial blood pressure when placed in the upper jejunum of animals anesthetized with pentobarbital. Clinically, the effective single oral dose has been found to vary from 0.03 to 0.08 mg/kg in hypertensive patients.[4]

Comparison of the effects of intravenous and intestinal administration in the anesthetized dog showed that the drug was more effective in producing fall of blood pressure by the intravenous route. In order to delineate the effect of slow absorption prolonged infusions were made. Slow intravenous administration became ineffective in the anesthetized dog below ¼ γ/kg/min. Effective doses by this method produced graded maintained hypotension. Comparison of the effects after splenic and femoral intravenous administration has not demonstrated inactivation of "Veriloid" by the liver.

Limited clinical trial of "Veriloid" has been accomplished by Freis and Wilkins. This will be reported separately. Certain individuals required as little as 2 mg or as much as 6 mg for single oral dose to produce blood pressure fall. Latency for full hypotension to be established was 2 hours.

Summary. A stable, reproducible and highly potent extract of *Veratrum viride* has been described. The name "Veriloid" has been given to this preparation. Methods for biological control of potency have been developed. In normal dogs the extract was hypotensive, bradycardic and emetic. Pharmacologic results suggest that the hypotensive action of the extract deserves trial in the treatment of human hypertension.

[4] Wilkins, R. W., and Freis, E. D., personal communication.

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17311. A Comparison of Desoxyribonucleic Acid Content in Certain Nuclei of Normal Liver and Liver Tumors.

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Direct chemical analyses of actively growing tissues, particularly tumors, for desoxyribonucleic acid have been reported by several investigators with, however, scant agreement. The percentage of desoxyribonucleic acid in nuclear material was found by Dounce[1] to be the same for Walker carcinosarcoma 256 and normal liver; in hepatoma 31 the percentage of desoxyribonucleic acid was lower than the normal. Brues, Tracy and Cohn[2] observed that the phosphorus and nitrogen content of hepatoma 31 and normal liver was the same per unit weight. On a similar basis, Davidson and Waymouth[3] and Schneider[4] recorded values for desoxyribonucleic acid that were higher in hepatomas than in normal liver.

Using the light absorption of fields of Feulgen stained nuclei, Stowell[5] also found

* Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This investigation was aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research.

[1] Dounce, A. L., *J. Biol. Chem.*, 1943, **151**, 235.

[2] Brues, A. M., Tracy, M. M., and Cohn, W. E., *J. Biol. Chem.*, 1944, **155**, 619.

[3] Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1944, **38**, 379.

[4] Schneider, W., *Cancer Res.*, 1945, **5**, 717.

[5] Stowell, R. E., *Cancer Res.*, 1946, **6**, 426.

TABLE I.
Relative Amount of Desoxyribonucleic Acid in the Nuclei of Normal Liver and of Liver Tumors.

Cell type	No. nuclei	(E) extinction coefficient	(A) nuclear area	(E) × (A) relative amount of DNA
		Slide 1		
Normal hepatic	10	.161–.187 (.177)	23	4.09 ± .14
Hepatoma	10	.237–.260 (.247)	16.5	4.09 ± .12
		Slide 2		
Normal hepatic	5	.208–.244 (.227)	23	5.22 ± .20
Cholangioma	10	.222–.265 (.225)	23	5.19 ± .23

higher values for the desoxyribonucleic acid in various tumors; here the results represented the average amount of desoxyribonucleic acid in many nuclei. The actual amount of desoxyribonucleic acid per single nucleus was not determined, nor was there any assay of the partition of the desoxyribonucleic acid into nuclear classes bearing different numbers of chromosomes.

Recent chemical determinations of the desoxyribonucleic acid in isolated nuclei by Boivin *et al.*,[6] Vendrely and Vendrely,[7] and Mirsky and Ris,[8] have demonstrated a remarkable species constancy in the desoxyribonucleic acid content of nuclei with the same number of chromosomes. In view of these findings, an attempt was made to establish the validity of this relationship for the presumably abnormal nuclei of tumors.

In the present study the amount of desoxyribonucleic acid in single nuclei of the same size has been compared in normal and tumor tissue utilizing the intensity of the Feulgen reaction. Precise data on the desoxyribonucleic acid content of single nuclei obtained by Mirsky and Ris[8] made it possible to use the Feulgen reaction as a measure of the relative amount of desoxyribonucleic acid per nucleus (Ris and Mirsky[9]).

The normal and tumor tissues studied were taken from the white rat. Liver tumors, hepatomas and cholangiomas, were induced by a diet of brown rice and carrot containing 0.06% p-dimethylaminoazobenzene as originally reported by Kinosita,[10] and were histologically identified according to the criteria described by Opie.[11] Tissue blocks were fixed in 10% formalin and cut at 10 μ thickness.

As elaborated elsewhere (Pollister and Ris;[12] Ris and Mirsky,[9]) the apparatus used in the measurements consisted of a Spencer monocular microscope with a mercury vapor arc as light source and a phototube and galvanometer stationed above a variable diaphragm in the image plane. The light absorption of individual nuclei stained by the Feulgen reaction was measured at a wave length of 546 mμ.

In Table I comparative measurements are shown of the smallest spherical nuclei in Feulgen stained paraffin sections of normal and tumor tissue mounted together on the same slide. The relative value for desoxyribonucleic acid per nucleus in the normal cell and in the hepatoma cell appears the same, as in slide 1. Similarly, slide 2 indicates the same desoxyribonucleic acid content per nucleus for the normal hepatic cell and the cholangioma cell.

Since the method is limited to the measurement of spherical nuclei, it was not possible to compare irregular bile duct nuclei with cholangioma nuclei directly, or to measure large nuclei of bizarre shape. Certain variations may be found in the values if the Feulgen technic varies slightly, as in Table I. Only sections on the same slide, therefore, are comparable.

[6] Boivin, A., Vendrely, R., and Vendrely, C., *Compt. Rend. Acad. Sci.*, 1948, **226**, 1061.

[7] Vendrely, R., and Vendrely, C., *Experientia*, 1948, **4**, 434.

[8] Mirsky, A. E., and Ris, H., *Nature*, 1949, **163**, 666.

[9] Ris, H., and Mirsky, A. E., unpublished data.

[10] Kinosita, R., *Trans. Jap. Path. Soc.*, 1937, **27**, 665.

[11] Opie, E. L., *J. Exp. Med.*, 1944, **80**, 231.

[12] Pollister, A. W., and Ris, H., *Cold Spring Harbor Symposia Quant. Biol.*, XII, 1947.

The results indicate that in the smallest spherical nuclei of liver tumors the amount of desoxyribonucleic acid is the same as in normal hepatic nuclei of similar size. Comparable results were independently obtained by Leuchtenberger,[13] who measured nuclei in transplanted mouse sarcoma. Considering the abnormal chromosome arrangements which are known to exist in neoplasms, (Boveri[14]) the presence in these tumors of nuclei with more or less nucleic acid cannot be excluded. The conflicting results of previous investigations may be attributed in part to the analyses of tissue samples containing different numbers and different sizes of nuclei.

Summary. The amount of desoxyribonucleic acid in single spherical nuclei of normal rat liver was compared with that of similar nuclei in tumor tissue by microphotometric determination of the intensity of the Feulgen reaction. It was found that the amount of desoxyribonucleic acid contained in nuclei of similar size is the same in hepatoma and cholangioma as in the normal liver.

[13] Leuchtenberger, C., personal communication.

[14] Boveri, T., The Origin of Malignant Tumors, Williams and Wilkins, Baltimore, 1929.

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17312. Excretion of Radiocalcium by Normal Rats.*

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The objectives of the present experiments were to determine the effect of various amounts of labeled calcium on the relative amounts of radiocalcium‡ excreted in the urine and feces, and to determine the distribution or radiocalcium in the tissues and contents of the gastrointestinal tract during the first few hours after the administration of a dose of labeled calcium.

Materials and methods. Young adult rats of the Sprague-Dawley strain were used. Immediately after the subcutaneous injection of a dose of labeled§ calcium each animal was placed in a separate wire metabolism cage over a urine-feces separator.[1] The animals shown in Table II were supplied with water only, but each of the other animals had access to both water and the stock diet, Purina Laboratory Chow, during the experimental period.

At the end of the experimental period the animals were dispatched by a blow on the head. In those instances in which the segments of the gastrointestinal tract and their contents were to be separately assayed for radioactivity, especial care was taken to remove the entire contents. The collected urine and cage washings, the feces, and the other samples were dried, and then dry ashed in an electric muffle at 550°C. In each instance the ash was dissolved in dilute hydrochloric acid and suitable aliquots transferred to small aluminum pans and dried under an infra red lamp. Dry sample weights were kept small (0.5 to 1.5 mg/cm^2 on sample pan surface) and comparable to the standards in order to minimize the error due to self-absorption of radioactivity. Suitably prepared standards and the samples were then assayed for radio-

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† Present address: Radioisotope Unit, Veterans Administration Hospital, Hines, Ill.

‡ The radiocalcium (Ca^{45}) used in these experiments was allotted by the United States Atomic Energy Commission, and supplied by the Monsanto Chemical Company.

§ 3 to 10 microcuries of Ca^{45}.

[1] Gross, I., and Connell, S. V. B., *J. Physiol.*, 1923, **57**, lx.

TABLE I.
Excretion of Radiocalcium after Injection of Various Amounts of Labeled Calcium.

No. of rats	Body wt., g	Dose of labeled Ca, mg	% of dose excreted	
			Urine	Feces
8	208 ± 12*	0.07	0.59 ± 0.25	12.60 ± 5.75
4	293 ± 30	0.21	0.61 ± 0.42	9.56 ± 1.13
3	224 ± 40	0.41	0.93 ± 0.65	11.36 ± 1.25
3	213 ± 12	2.50	5.90 ± 1.75	15.17 ± 1.14
3	190 ± 8	4.90	2.95 ± 0.94	12.46 ± 1.10
6	211 ± 30	7.00	2.49 ± 1.55	9.41 ± 5.40
10	233 ± 35	14.00	6.05 ± 2.15	7.74 ± 4.50

* Standard deviation of the mean.

activity by using a scale-of-sixteen Geiger-Muller counter, equipped with a thin mica window (1.8 mg/cm^2) tube.

Results and comments. The data summarized in Table I show that under the conditions of our experiments subcutaneously administered radiocalcium was excreted mainly in the feces. Variations in the relative amounts of radiocalcium excreted in the urine and feces were large for each dose of labeled calcium injected, but the results indicate clearly that the amount of calcium in the dose was an important factor in determining the relative amounts of radiocalcium excreted in the urine and feces. As would be expected, retention of the radiocalcium varied widely and probably reflects differences in the rate of absorption of the labeled calcium, or differences in the calcium requirement of the animals.

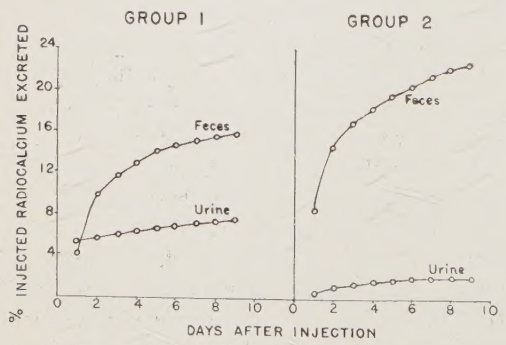

FIG. 1.

Group 1 consisted of 4 rats (avg wt. 265 g), each of which received two subcutaneous doses of 7 mg of labeled calcium within the first hour of the experimental period. Group 2 consisted of 4 rats (avg wt. 220 g), each of which received a single subcutaneous dose of 0.07 mg of labeled calcium at the beginning of the experimental period.

Fig. 1 shows graphically the excretion of radiocalcium by 2 groups of rats during the 9 days following administration. The animals of Group 1, each of which received an injection of 7 mg of labeled calcium at the beginning of the experimental period and a second injection of 7 mg at the end of the first hour, excreted 5.2% of the administered radiocalcium in the urine and 4% in the feces during the first 24 hours. Thereafter the amounts of the radiocalcium which appeared daily in the feces exceeded the amounts excreted in the urine. Summation of the fractions of the administered radiocalcium found in the urine and feces during the 9-day experimental period reveals that approximately 7.27% of the dose was excreted in the urine and 15.9% in the feces.

The results obtained for the animals of Group 2, Fig. 1, show that when the quantity of labeled calcium injected was only 0.07 mg the average amount of the administered radiocalcium excreted in the urine during the first 24 hours was only 0.66%, while the average amount excreted in the feces was 8.49%. In the case of this group of animals approximately 2.10% of the dose of labeled calcium was excreted in the urine and 22.75% in the feces during the 9-day experimental period. From these experiments (Fig. 1) it is apparent that the gastrointestinal tract was the principal route by which the labeled calcium was excreted. Furthermore, it appears that only when a plethora of the labeled calcium was being eliminated during the first 24 hours did the kidneys respond by excreting a large fraction of the administered radiocalcium.

It is generally agreed that a considerable amount of excreted calcium enters the lumen

TABLE II.
Distribution of Radiocalcium in Tissues and Contents of Gastrointestinal Tract.

Rat No.	Body wt., g	Hr after dose of 7 mg labeled Ca	% of dose recovered from tissues				% of dose recovered from contents			
			Stomach	Small intestine	Cecum	Colon	Stomach	Small intestine	Cecum	Colon
1	205	1	0.14	0.40	0.01	0.08	0.23	1.79	0.29	0.12
2	190	1	0.11	0.60	0.02	0.11	0.12	0.91	0.10	0.06
3	204	1	0.10	0.69	0.03	0.07	0.15	0.92	0.14	0.16
4	185	1	0.20	0.40	0.02	0.07	0.11	1.10	0.12	0.07
Avg	196		0.14	0.52	0.02	0.08	0.15	1.18	0.16	0.10
5	178	3	0.17	0.49	0.03	0.14	0.09	1.24	0.57	0.35
6	196	3	0.12	0.63	0.04	0.12	0.05	1.23	0.17	0.13
Avg	187		0.14	0.55	0.03	0.13	0.07	1.23	0.37	0.24
7	190	6	0.11	0.44	0.03	0.13	0.05	0.41	1.71	0.17
8	187	6	0.12	0.57	0.01	0.10	0.08	0.48	1.17	1.57
9	186	6	0.14	0.71	0.04	0.28	0.03	0.41	1.41	0.14
Avg	188		0.12	0.57	0.03	0.17	0.05	0.43	1.43	0.63

of the intestine as a constituent of the intestinal secretions[2] and the bile,[3] and that this calcium, supplemented by a fraction of the dietary calcium is reabsorbed. Evidence of an active excretion of calcium through the wall of the small intestine,[4] or the colon,[5–7] however, has not been equally acceptable.

In our experiments (Table II) the employment of radiocalcium has made possible the orientation of various portions of the gastrointestinal tract with respect to the excretion of calcium. The distribution patterns of radiocalcium in the tissues and contents of the gastrointestinal tracts of animals, which were sacrificed at 1, 3, and 6-hour intervals after the subcutaneous injection of 7 mg of labeled calcium, are shown in Table II. Of particular interest was the relatively large fraction of the excreted radiocalcium found in the contents of the small intestine and the smaller fractions recovered from the contents of the cecum and colon at the 1-hour interval. In Rats 1 and 2 the small intestine was severed at the midpoint and the tissues and contents of the two halves separately assayed for radioactivity. The tissues of the upper and the lower half of the small intestine of Rat 1 contained 0.24 and 0.16% of the administered radiocalcium, respectively, whereas the contents of the upper and lower half contained 0.43 and 1.36%, respectively. In the case of Rat 2 the tissues of upper and lower half of the small intestine contained 0.28 and 0.32% of the administered radiocalcium, respectively, and the contents 0.13 and 0.78% respectively.

Unfortunately separate assays were not made on the tissues and contents of equal parts of the small intestine of Rat 5 or 6. However, such assays were made on Rats 7, 8, and 9. In these animals approximately the same amounts of radio-activity were found in the tissues of the upper and lower half of the small intestine, but in 2 instances the amounts of radiocalcium in the contents of the 2 segments differed appreciably. In Rat 7 the contents of the upper and lower half of the small intestine contained 0.12 and 0.29% of the injected radiocalcium, respectively, and in the case of Rat 9 the fractions of the administered radiocalcium recovered in the 2 segments were 0.09 and 0.32%, respectively. The contents of the upper and lower half of the small intestine of Rat 8 contained 0.20

2 Logan, M. A., *Physiol. Rev.*, 1940, **20**, 522.

3 Greenberg, D. M., and Troescher, F. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 488.

4 Walsh, E. L., and Ivy, A. C., PROC. SOC. EXP. BIOL. AND MED., 1928, **25**, 839.

5 Bergeim, O., *J. Biol. Chem.*, 1926, **70**, 51.

6 Cowell, S. J., *Biochem. J.*, 1937, **31**, 848.

7 Kosman, A. J., and Freeman, S., *Fed. Proc.*, 1943, **2**, 16.

and 0.28% of the injected radiocalcium, respectively.

It is very doubtful that the major portion of the excreted radiocalcium found in the lower half of the small intestine could have resulted from the failure of the upper half to reabsorb secreted calcium. Duckworth and Godden[8] have observed in the rat that the net absorption of ingested calcium is not affected by a diet containing 30% of fiber. Since such a diet should cause a great increase in the intestinal secretions, it would appear that the efficiency of reabsorption of secreted calcium is very high in the rat. Therefore, we are inclined to regard that fraction of the excreted radiocalcium found in the lower half of the small intestine as indicative of an active excretion of calcium through the wall of the intestine. In this connection, the extreme rapidity with which the fecal excretion of calcium has been shown to occur is of great interest. Norris and Kisieleski[9] recovered 5 to 8% of a dose of labeled calcium from the contents of the intestines of rats one minute after intravenous administration.

The presence of relatively small fractions of excreted radiocalcium in the contents of the cecum and colon at the 1 hour interval (Table II), and the larger amounts in the contents of these segments at later intervals, suggest that the bulk of the excreted radiocalcium entered the small intestine at points above the ileocecal valve. This observation is in accord with evidence that has been advanced[10] that there is little or no active excretion of calcium into the large intestine.

Summary. 1. The excretion of subcutaneously administered radiocalcium has been studied in normal rats.

2. The amount of labeled calcium injected appeared to be an important factor in determining the relative amounts of radiocalcium excreted in the urine and feces. When the quantity of labeled calcium injected was increased from 0.07 mg to 14 mg the fraction of the excreted radiocalcium which appeared in the urine in the first 24 hours increased from a relatively small value to a value approaching or slightly exceeding the amount of radiocalcium excreted in the feces. During a 9-day experimental period the amount of radiocalcium excreted in the feces, when either 0.07 or 0.14 mg of labeled calcium was administered, greatly exceeded the amount excreted in the urine.

3. The distribution of radiocalcium in the tissues and contents of the stomach, small intestine, cecum, and colon was determined at 1, 3, and 6-hour intervals after the administration of a dose of labeled calcium. The results of these experiments indicate that there is little or no active excretion of calcium through the wall of the large intestine.

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